

**ECOLOGY AND THERMAL INACTIVATION OF MICROBES  
IN AND ON INTERPLANETARY SPACE VEHICLE  
COMPONENTS**

**Combined Thirty-third and Thirty-Fourth Quarterly Reports of Progress**

**Order No. W-13411**

**April 1, 1973 - September 30, 1973**

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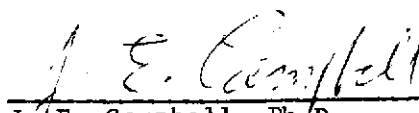
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Investigation on the Mechanism of Thermal Inactivation  
of Bacterial Endospores under Conditions  
of High Relative Humidity

A Thesis submitted to the

Department of Civil and Environmental Engineering  
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in partial fulfillment of the  
requirements for the degree of

Master of Science

1974

by

Antolin L. Reyes

B.S. Ohio State University 1957

## INTRODUCTION

Thermal inactivation of bacterial spores under conditions of high humidity has been of interest as a means of gaining insight into the mechanisms involved. This problem has been under investigation for sometime in our laboratory by Antolin L. Reyes. In addition to providing information for the project, his work was suitable also for a thesis in partial fulfillment of a Masters of Science degree, and is herewith presented as the Thirty-third Quarterly Report of Progress.

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## Introduction

The basic purpose of this study is to show that the changes that occur in the heat sensitivity of bacterial spores exposed to elevated temperature and high humidity is identical to the changes occurring in the sensitivity of spores undergoing germination. The results should provide some answers on the mechanism of thermal inactivation under these conditions of high humidity and temperature.

Preliminary work in our laboratory showed survival curves of Bacillus subtilis var. niger spores exposed to moist heat did not follow a first order reaction. Several investigators also obtained wet heat survival curves that were not log linear (1), (8), (11), (13), (19), (25). Contrastingly, spore inactivation curves under dry heat as proposed by Rahn, 1945 (32) and Watkins and Winslow, 1932 (40) followed a first order reaction.

For the past several years, researchers of the National Aeronautics and Space Administration were engaged in studies on dry heat inactivation of bacterial spores. Since spores are very highly resistant to heat, the findings and data were useful in establishing guidelines for sterilization processes on interplanetary vehicles and components (34).

However, very little work has been done in identifying the mechanism of thermal inactivation of bacterial spores under "moist" or "dry" heat. Since very little data has been reported in this area this investigation was undertaken and the results of this study are presented.

This investigation included several areas of study involving establishment of experimental conditions relating to spores loss of heat resistance, loss of optical density as a measure of the rate and extent of germination on spore suspensions, events occurring during germination and correlating these events with phase darkening (refractility and non-refractility of spores), stainability characteristics of heat and non-heat treated spores, morphological characteristics of spores by scanning electron microscopy and studies on swelling of spores by an increase in packed cell volume.

## Review of Literature

The conventional method of following the kinetics of germination of a spore suspension is by exposing the spores to heat and by determining the effect of the heat treatment on viability by means of plating. The effect of heat on bacterial spores appears complex. Besides heat lability, other criteria of germination are darkening under phase contrast microscope; decrease in absorbancy by optical density measurements; onset of stainability, depolymerization, and excretion of spore constituents; cytological changes; increase in permeability and spore volume.

A spore, because of its structure (page 9) and its change from a dormant spore to a vegetative body (page 10), provides research workers and students with an interesting means for its study. Germination systems are illustrated on pages 11 and 12.

What is germination?

Germination is essentially the conversion of a resistant and dormant spore into a sensitive and metabolically active form. During germination spores also lose their characteristic resistance to dessication, pressure, vacuum, ultraviolet and ionizing radiations, antibiotics, and other chemicals.

Powell (30) used heated single spores germinating in microscope slide cultures, and showed that loss of heat resistance of individual spores occurred rapidly and completely.

Robinson (33) reported that there are differences of opinion on what should be regarded as the moment of germination.

In 1957, Knaysi (15) reported that heat sensitivity is a criterion of germination because it takes place before the process is completed.

Physiologists date this event from the moment that more or less coincides with the onset of the first visible changes when the spore loses its heat resistance and dryness of its interior, and begins to secrete some of its substance to the medium.

Other researchers believe that there has not been germination until the new bacterium has slipped out of the spore coat. Germination is a lengthy and complicated process.

In viable resting spores examined in water, coat, cortex, and core can be distinguished from each other only with difficulty or not at all.

The clear separation of the spores early in germination and while the core is still shiny suggest that water is taken up at this time by the cortex. As germination proceeds, the cortex becomes thinner and fades from view. This corroborates the disintegration of the cortex found in electron micrographs of sections of germinating spores of Bacillus megaterium.

Evans and Currans in 1943 (6, 7) showed that relatively mild heating of the spores of mesophilic aerobes hasten their germination. Their study was extended on a group of thermotolerant aerobes isolated from commercially canned evaporated milk that had spoiled.

#### Medium and Germination

Holzmuller (1909) reported spores of Bacillus mycoides did not germinate in distilled water and in physiological NaCl solution.

Knaysi (1945) (14) reported also that spores of strain C<sub>2</sub> of B. mycoides did not germinate in solutions of potassium phosphate, potassium nitrate, lactose, potassium phosphate, and nitrate plus lactose, but they did germinate in a solution of glucose without nitrogen sources.

Pulvertaft and Haynes in 1951 (31) reported that although 14 amino acids in an arbitrary mixture, DL-alanine, L-tyrosine, DL-cystine, DL-tryptophan, and methionine did not have stimulative effect on the germination on Bacillus subtilis.

#### Changes in Resistance

Loss of resistance which depends on the structural integrity of the of the spore coats to high voltage, or to enzymes and metabolic inhibitors, occurs later during dissolution of the cortex or emergence of the new vegetative cell from the spore coats. The extent of fall in heat resistance during germination varies according to the heat resistance of the spores (which can cover a range of about  $10^5$ -fold<sup>6</sup>) but is large and normally ranges between about  $10^3$ -fold and  $10^6$ -fold.

The isolation of DPA from bacterial spores (27, 28) immediately stimulated research into its involvement in determining heat resistance. DPA has been thought to be associated with the cytoplasm (18) and not the cortical zone, since spores depleted of DPA by autoclaving retain the latter, but its true location is not clear. In B. cereus T resistance varied directly with DPA content but in Cl. roseum and B. coagulans or between species, this simple relationship was not found.

Calcium had previously been thought to play an important role in heat resistance (37) but again the content in different spores did not correlate well with resistance (5).

In a study of 20 spore preparation, Murrell and Warth (24) found a significant relationship between calcium and heat resistance, whereas magnesium showed a significant inverse relationship.

The development of resistance has also been related to the appearance in the spore of cystine rich materials (39) although the determination in

these studies was not precise owing to spores forming in chains.

Other workers have approached heat resistance by studying the resistance of individual cell components.

Any explanation of resistance must take into account the enormous range of resistance of spores to heat (about  $10^5$ -fold between C. botulinum type E and B. stearothermophilus) as well as small differences in resistance of spores of different species, and different strains. Yet these spores have remarkably similar structures, and very similar compositions.

#### Breaking of Dormancy

The appearance of enzymic activity during germination results mainly from the rapid unmasking or activation of preexisting enzymes rather than from new synthesis. Dormancy is not necessarily maintained by the same mechanisms that maintain spore resistance, but there is evidence that calcium dipicolinate, murein in the spore cortex and the state of hydration of the spore core are all involved.

Increase in respiratory activity during germination is very great, as compared to the respiration rate of inactivated ungerminated spores, which is so low that it cannot be detectable, using sensitive radioactive tracer techniques (9).

#### Depolymerization and Excretion of Spore Constituents

During germination spores excrete material amounting to 30% of their dry weight. The exudate is principally composed of calcium, a roughly equimolar amount of dipicolinic acid, and fragments of depolymerized murein (28). In addition, small amounts of amino acids, small peptides, and proteins are present in spore germination exudate. The exuded calcium may originate from the core, which is thought to be rich in this mineral in the dormant spores (16) or from the cortex.



"Spore peptide" in germination exudate certainly originates mostly from murein in the spore cortex, which is probably depolymerized during germination of enzymes like the S-enzymes of Strange and Dark (35).

#### Cytological Changes

The principal cytological change accompanying germination is in the structure of the cortex, which almost completely disappears during germination of some organisms (e.g., B. cereus, B. megaterium (21), B. anthracis). At the same time, or closely following the changes in the cortex, elements of the cytoplasm become more distinct. In particular, the nuclear areas which are indistinct in sections of dormant spores become less electron dense and more obvious. In B. subtilis spore ribosomes become distinct and small vesicles appear in the cytoplasm but attached to the core membrane. Swelling accompanies germination so that the exosporium enveloping spores may be rapidly filled although remaining intact (23).

#### Optical Density Changes

The optical extinction of visible light by a spore suspension decreases during germination, commonly by about 60%, but its extent differs with different organisms. The observed fall in extinction is due mostly to the excretion and solubilization of dry matter from the germinating spores and the consequent fall in refractivity and light absorption by individual spores.

#### Phase Darkening

During germination, spores change from bright to dark as viewed by phase contrast optics at about the same time as heat resistance is lost. Phase darkening occurs because of the loss of refractive index of spores during germination. The fall in refractive index accompanying germination probably results from the combined excretion of dry matter, slight swelling

and possibly the redistribution of water within the spore during germination.

#### Onset of Stainability

Clean dormant spores are essentially unstainable except at their peripheries unless pretreated by heat or acids, whereas germinated spores are readily stained by simple stains. Stainability was first used by Powell (26, 29) and has been used since along with phase-contrast microscopy and the measurement of extinction changes as a rapid method of estimating the percentage germination in a spore suspension. Germinating spores may become stainable because of breaching of a permeability barrier. Stainability may accompany the unmasking of reactive groups in the spore.

It is assumed that the majority of reactive groups in the dormant spore are unavailable for reaction with stains because they are chemically blocked. The most likely blocking agents would be dipicolinic acid (for basic groups) and calcium (for acidic groups) or perhaps 1:1 calcium dipicolinate molecule.

#### Increase in Permeability

The hypothesis that dormant spores have a low water content and are impermeable to water would presuppose that a large increase in permeability occurs during germination. However, the "anhydrous spore" hypothesis has been shown untenable both on theoretical grounds and by actual measurement of the water content of spores. Such permeability changes that do occur in spores during germination evidently involve only certain parts of the spore (e.g., the core) and the accessibility of functional groups. Black and Gerhardt (2) measured small increases in the total space in spores accessible to water, glucose, and stains which occurred

during germination of spores of B. cereus T but pointed out that the small overall changes measured did not exclude the possibility of larger local changes occurring within the spore.

#### Increase in Spore Volume

Following germination in a medium which can support outgrowth, spores may approximately double in volume before the new cell emerges from the spore coats. This enlargement can be seen microscopically and can be measured as an increase in packed cell volume (12) and is mostly due to growth, i.e., synthesis of new cell material.

Swelling accompanying germination was recognized by many workers. In these studies, spores at the same time the volume increased the germinating spores became more spherical. The increase in spore breadth during germination is relatively greater than the increase in length. Such a sudden increase in size and change in shape suggests that the spore coat is or quickly becomes plastic during germination, perhaps because of action of lytic enzymes or disulphide reductases (39). Rehydration and expansion of the core accompanying release of pressure exerted by a contractile cortex could certainly cause germinating spores to assume more spherical shapes.

#### Kinetic Model for Bacterial Spore Germination

Woese, et al. (41) proposed a model for the kinetics bacterial spore germination based on a simple enzyme model. He assumed that germination occurs when the level of some substance, P, in the spore reaches a critical value,  $P_c$ , and that substance P is produced by a "germination enzyme" contained in the spore. He further assumed that to a first approximation, germination enzymes are distributed among the individual spores in a spore population according to a simple Poisson distribution. Thus, the

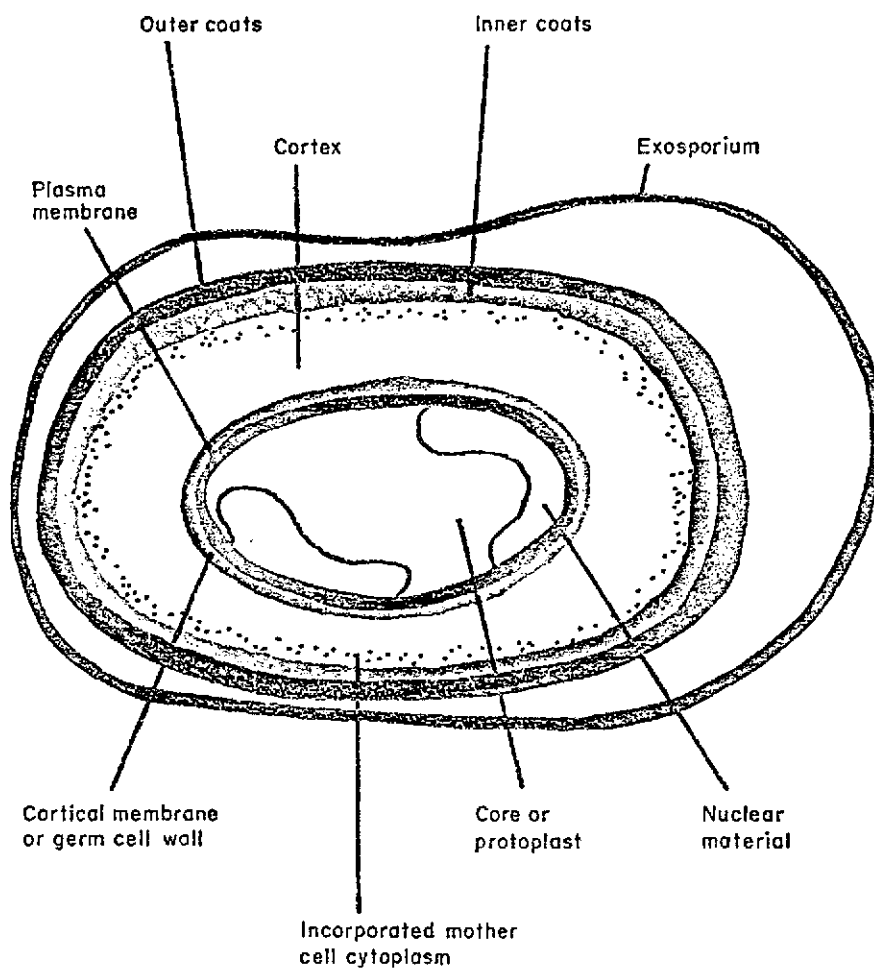
spore population can be divided theoretically into subpopulations each characterized by having a characteristic number,  $n$ , of enzymes per spore in every individual of the subpopulation.

There are commercial reasons for gaining an insight into the mechanisms of spore resistance to heat. For example, if some food processes could be made less severe without loss of microbiological stability, not only would time and costs be reduced, but quality would also be improved and new markets opened.

There is the trend in public preference for foods less and less damaged by over-cooking, and for foods that are less acid or salty. In addition, the growing control of chains of food distribution and the increasing use of refrigeration, encourage the production of non-sterile foods with correspondingly limited storage life. Again, all this points to attempts to reduce processing treatments, while maintaining accepted standards of security from spoilage and from risk of food-poisoning.

Mikolajcik (22) reported the significance of spores of Bacillus species because of their ability to survive conventional heat treatments used in the dairy foods industry. The multiplication of these spores in many dairy food products under appropriate conditions subsequently create economic and potential public health problems.

Historically, endospores have played a major role in bacteriology. Because of spores exceptional heat resistance, crucial experiments have to be devised. Today, spores have been used as indicator organisms on sterilization processes in the laboratory and in industry. The challenge of insuring a high degree of control of sporeformers in processed foods is still with us and a detailed understanding of the mechanism of spore death and dormancy is necessary.



*Frontispiece: Diagrammatic representation of the structure of the bacterial spore*

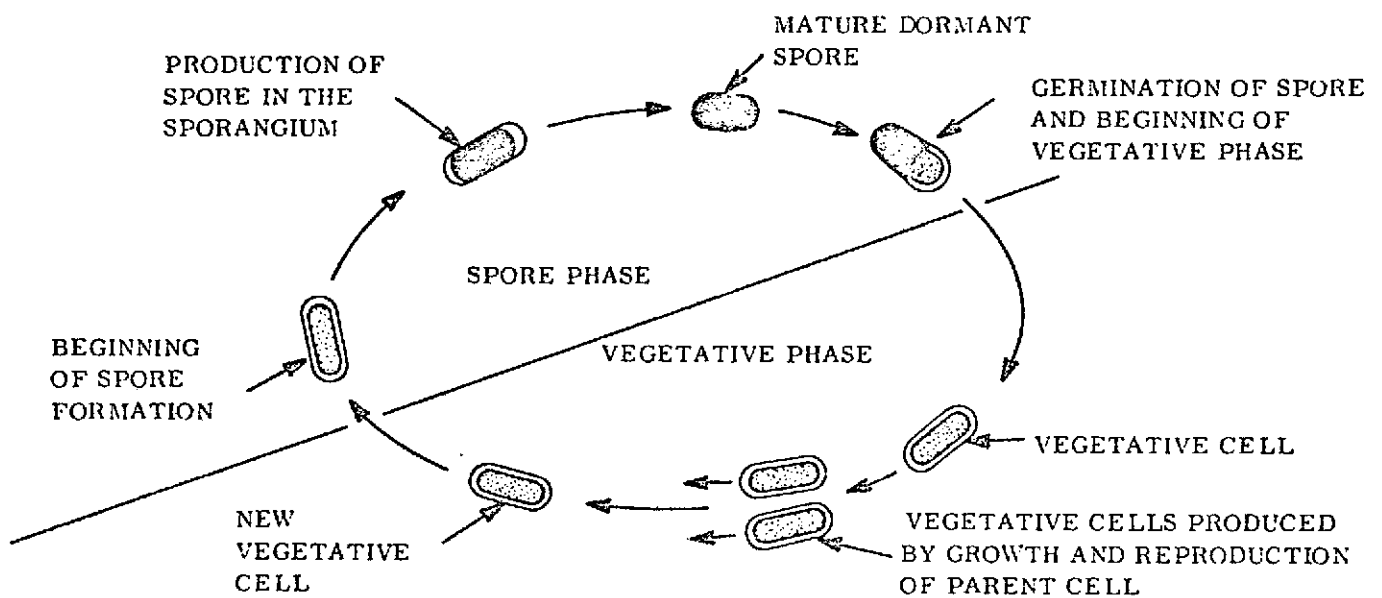


Figure 3-5. Spore Germination  
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## Germination and the problem of dormancy


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TABLE 2—continued

Germination system	Responsive spores	Qualifying remarks
<i>Enzymic germinants</i>		
Subtilisin	<i>B. subtilis</i> and some other species	<i>B. subtilis</i> protease, which can germinate spores of a number of species. Can partly degrade spore coats. Probably acts via formation of L-alanine.
Initiator protein	<i>B. cereus</i> , <i>B. licheniformis</i>	Extracted from <i>B. cereus</i> spores. Germinates heat activated spores with co-factors L-alanine + NAD. May act via formation of low MW riboside germinants.
Lysozyme	Probably all spores	With few exceptions spores must first be sensitized by oxidizing or reducing agents. Lysozyme then causes germination-like changes. These are followed by partial lysis in those organisms which have lysozyme sensitive vegetative cells (e.g. <i>B. subtilis</i> and <i>B. megaterium</i> , but not <i>B. cereus</i> ).
Spore lytic enzyme	<i>B. cereus</i> , <i>B. subtilis</i>	Extracted from <i>B. cereus</i> spores. Acts like lysozyme (above).
<i>Physical germinants</i>		
Abrasion	Probably all spores	e.g. Grinding with powdered glass can induce germination-like changes, with retention of viability.
Crushing	Probably all spores	e.g. Pressure of microscopic nosepiece on cover-glass or scraping with a wire loop can cause sufficient distortion of spores to initiate germination-like changes.
Hydrostatic pressure	Probably all spores	Causes rapid germination of least dormant spores. Less effective on super-dormant spores. Very temperature dependent. Synergistic with amino acid germinants at the lower pressure-temperature regions.

TABLE 2  
Principal germination systems for bacterial spores

Germination system	Responsive spores	Qualifying remarks
<i>Nutrient germinants</i>		
L-Alanine	Many; including most strains of <i>B. cereus</i> , <i>megaterium</i> , <i>subtilis</i> , <i>pinus</i> , <i>macerans</i> , <i>coagulans</i> , <i>stearothermophilus</i> and some <i>Clostridium</i> spp.	Often replaceable by L- $\alpha$ -amino butyric acid, L-cysteine, L-valine; less often by other amino acids. Inhibited by D-alanine. Effective levels vary greatly for different strains and species.
Other amino acids	As above, but more restricted range of organisms	Usually less active than L-alanine. Not inhibited by D-alanine. Leucine has a specific potentiating effect with some strains.
Purine ribosides	<i>B. cereus</i> , <i>B. megaterium</i> strains and others; not <i>B. subtilis</i> , <i>B. coagulans</i> , <i>B. stearothermophilus</i> ; not <i>Clostridium</i> spp.	In decreasing order of activity; inosine, adenosine, guanosine, xanthosine (relatively inactive). May cause germination alone, but most dramatically potentiate the action of low levels of L-alanine and other neutral amino-acids. Not inhibited by D-alanine.
Purine bases	Certain strains of <i>B. macerans</i>	Adenine, 2,6-diaminopurine: unique to these strains so far.
Lactate	<i>Cl. bifermentans</i> , <i>Cl. tetani</i>	So far only described for <i>Clostridium</i> spores. Effective with other germinants rather than alone.
Nicotinamide	<i>Cl. tetani</i>	Novel implication of a vitamin: effective with other germinants. Nicotinic acid ineffective.
Sugars	<i>B. megaterium</i> , some <i>B. subtilis</i> and <i>Clostridium</i> spp.	Few organisms respond to glucose alone. Sugars may potentiate action of other germinant. Various sugars apart from glucose, and amino sugars active. Usually replaceable by other germinant systems.
<i>Non-nutrient germinants</i>		
Ions	Some <i>B. megaterium</i> and <i>B. polymyxa</i> strains	These spores are unusual in that substantial germination is caused by various ions alone. However, most spores have specific Na <sup>+</sup> or K <sup>+</sup> requirements, and a general requirement for ions (optimally equiv. to c. 100 mM NaCl) for germination.
Bicarbonate	Some <i>Clostridium</i> spp.	Often reported to potentiate strongly germination initiated by nutrient germinants or in complex media at concentrations c. 50 mM. Ineffective with normal <i>Bacillus</i> spores, but potentiates germination of <i>B. cereus</i> T DPA-less mutant spores.
n-Dodecylamine	Probably all spores	Causes rapid germination at micromolar concentrations, and even in extreme non-physiological environments. Some other surfactants are active but less effective. Germinated spores remain viable if surfactant is quickly quenched.
Calcium dipicolinate (CaDPA)	Most spores	Most effective as 1 : 1 CaDPA chelate at near saturation levels (i.e. c. 30-40 mM). Will often germinate otherwise dormant or damaged spores.

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## Materials and Methods

### Production of Spores

The test organism was Bacillus subtilis var. niger spores. Actively growing vegetative cells were grown on an agar surface medium (0.25% Sutz filtered glucose; Casamino acids (Technical grade), 0.25%; yeast extract, 0.5%,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.001%;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0014%; agar 3%) in 6-liter Povitsby bottles and incubated for seven days at 35° C during which time spores were produced. The spores were washed from the surface with double distilled water shaken with glass beads, filtered through cotton suspended in distilled water and held at 45° C in a water bath overnight. The heated suspension was washed five times in double distilled water (4080 g for 30 minutes at 5° C) and was suspended and stored in 95% ethyl alcohol at 5° C.

### Determining Thermal Resistance

The heat resistance of spores was determined by the conventional plating technique. For each experiment the working suspension was sonified prior to making the appropriate concentration of a spore suspension. Aliquots of aqueous spore suspensions (approximately  $1 \times 10^6$  spores per ml) were placed in 16 X 125 mm screw capped culture tubes, heated at appropriate time intervals and temperature, then cooled for 15 minutes in a 4° C circulating water bath. Tenfold serial dilutions were made of each sample and plated using tryptone glucose beef extract agar. The plates were incubated at 35° C for two days and counted.

Recording counts. Colonies growing on plates were counted. To compute viable organisms per ml the total colonies per plate were multiplied by the reciprocal of the dilution used. The results were plotted on a semilog paper, i.e., the number of viable organisms on the ordinate and the time on the abscissa.

## Growth Studies

Measurements of turbidity were measured by a Turner Fluorometer Model 110 converted into a Nephelometer. This instrument measures the amount of light scattered by extremely dilute suspensions at a bacterial cell range of  $1 \times 10^3$  cells per ml. Heated spore samples were cooled at  $4^\circ \text{C}$  for 15 minutes unless otherwise specified and were subcultured in Erlenmeyer flasks containing tryptone glucose beef extract broth to give a spore concentration of about  $1 \times 10^4$  spores per ml or  $1 \times 10^5$  spores per ml. A magnetic stirring bar was placed in each flask and the flask was placed on top of a "magnestir" plate in a  $35^\circ \text{C}$  incubator. Samples were withdrawn at appropriate time intervals, placed in cuvettes and a turbidity reading taken.

## Optical Density

A sonified spore suspension was diluted in phosphate buffered water unless otherwise specified to give a concentration of about  $1.0$  to  $1.5 \times 10^8$  cells/ml. The spore suspension was equally divided into a series of 16 X 125 mm tubes (screw capped) and heated in a thermostatically controlled oil bath at the appropriate temperature. An unheated control spore suspension was used for every experiment. The unheated or heated medium was either in phosphate buffered water, tryptone glucose beef extract broth or phosphate buffered water plus subsequent transfer in tryptone glucose beef extract broth 1:1 (V/V). Initial optical density readings were taken following the heating process. Rate and extent of germination were taken by incubating tubes of spore suspensions at  $35^\circ \text{C}$ .

Germination was measured by the decrease in optical density of the spore suspension in a Bausch and Lomb Spectronic-20 colorimeter at 625 nm. The percentage decrease in optical density was calculated as follows:

$$\left( \frac{\text{initial optical density} - \text{final density}}{\text{initial optical density}} \right) \times 100$$

#### Packed Cell Volume Studies

Cl sonified spore suspension was diluted in phosphate buffered water, unless otherwise specified, to give a concentration of about  $1.0 \times 10^9$  cells/ml. The spore suspension was dispensed equally into each of 16 X 125 mm glass tubes (screw capped). The tubes of spore suspension were heated in a thermostatically controlled oil bath and temperature was monitored by thermocouples immersed inside a control tube and in the oil bath by a thermo-recorder.

PCV measurements were done by the method of Hitchins 1963 (12). Heated and non-heated spore suspensions were dispensed in Servall centrifuge tubes and spun for 30 minutes at 2000 RPM (International Centrifuge PR<sub>2</sub> model equipped with a horizontal head). The supernatant was removed with a 5 ml pipette and the pellicle reconstituted by adding 0.1 ml of Octanol 1 solution into each tube to prevent foaming. Samples were drawn into each capillary haematocrit tube, flame sealed with a haematocrit tube sealer and centrifuged for 5 minutes.

PCV

at 15,000 RPM.

The length of the pellet and the total liquid length in each capillary tube were measured with a Glogaus Vernier Caliper #12.

The packed cell volume was expressed in percentage by using the formula:

$$\text{PCV} = \frac{\text{length of pellet (mm)}}{\text{length of pellet plus supernatant fluid}} \times 100$$

### Phase Darkening

Determination of extent of germination was done by phase contrast microscopy Pulvertaft and Haynes, 1951, (31 ) and Wolf, et al., (42).

Heat fixed smears were examined on the following classes (1) ungerminated, fully refractile spores (2) partial and full germination, spores with a darkened periphery area or partial refractility and completely darkened area, both the center and periphery respectively. Spores were counted (500 spores) per sample and a percentage of each category determined.

A loopful of a heated or non-heated sample was spread on a clean 1 X 3 inch milk slide in a circular centimeter area. The sample was spread evenly with a rotary motion to cover the area evenly and the film heat fixed quickly over a bunsen burner. The film was treated with a 2:1 (V/V 95% alcohol glacial acetic acid mixture for 5 minutes. The mixture was poured off and rinsed with water. The prepared smear was mounted in water sealed under a cover slip (No. thickness; 18 mm, square) with Vaspar. The preparation was examined with the immersion lens under a 2 in. microscope equipped with 100 X objective (Neofluar phase 100/1.30 Zeiss). About 500 spores were counted and a percentage taken of each category mentioned earlier.

### Phase Darkening

Photomicrographs were also taken on heated and heat treated spores on panchromatic film (Kodak plus X ASA 125) and on a high speed Kodak Ektachrome ASA 125 film.

### Stainability

The differential staining procedure of Bartholomew and Mittmer's "Cold" Method (43) using malachite green and safranin was used to determine the percentage of ungerminated and germinated spores.

Uehara and coworkers (38) used stain uptake as a criterion of germination. In our study a differential stain was employed using 7.6% malachite green as a primary stain and 0.25% safranin as the counterstains. Primary stain was lost at the same rate as partial darkening of the spore (s).

### Scanning Electron Microscopy

Spores suspended in phosphate buffered water (about  $1 \times 10^9$  per ml) were heated in three 16 x 125 mm screw capped tubes at 90° C exposed at 0, 30, and 240 minutes. Samples were plated for viability and the remaining portion placed in 2X Millionigs phosphate buffer solution (personal communication (20) and fixed in 2% glutaraldehyde solution. The organisms were observed later by SEM and photomicrographs taken.

## Results and Discussion

### The Effect of Temperature on Spores Suspended in Phosphate

#### Buffered Water

The characteristics of survival curves on all temperatures studied (85, 90, 95, and 100° C) of spores suspended in phosphate buffered water is shown in Figure 1. As expected, the survival curves of spores exposed at these conditions showed an induction period at the early stage of heating followed by a sudden decline of the number of viable organisms. The length of the induction period is reflected in the differences in temperature prior to rate of destruction.

### The Effect of Heat Treatment on Spores Suspended in Tryptone Glucose Beef Extract Broth

When spores were heated in tryptone glucose beef extract broth at 90° C, the survival curve showed a similar pattern compared to the survival curve of spores suspended in phosphate buffered water at the same temperature. This is shown in Figure 2. However, the effect of the suspending medium is significantly different on the rate of the inactivation of the spores. This effect is further magnified when spores were pretreated in tryptone glucose beef extract broth for 60, 150, 210, 270 and 330 minutes at 25° C before heating at 90° C. The result is shown in Figure 3.

### Equivalency of Heat Treatment

Equivalency of heat treatment was attempted by picking a "time point" along the induction period on spores heated at 90° C in phosphate buffered water and spores heated at 90° C in tryptone glucose beef extract broth. The effect on the growth rate of the spores is shown by subculturing the

heated samples in tryptone glucose beef extract broth and following the growth rate by turbidimetric measurements. The growth curves are shown in Figure 4. The growth rate of the spores on the two treatments are essentially the same.

#### The Effect of 90° C on Spores Suspended in Phosphate Buffered Water at Different Exposure Times

With these previous findings I proceeded in my investigation to determine the heat sensitivity of the spores. The spores were suspended in phosphate buffered water at 0, 10, 20, 30, 40 and 50 minutes at 90° C. Turbidimetric measurements were taken and the results are shown in Figure 5. The results are quite unexpected. The growth rate of spores showed a progressive lag as heat exposure was extended from zero to 50 minutes. Could cooling the samples at 4° C after heating have an effect on the germinating spores? So another experiment was set up which is similar to the previous one except heated samples were immediately dispensed in appropriate amounts in a 35° C tempered tryptone glucose beef extract broth. The results are shown in Figure 6. The growth rates were accelerated compared to the growth rates shown in Figure 5. However, what is significant is that the lag in growth rate on spores treated for 20 and 30 minutes were significantly greater than non-heat treated spores. These results suggest that possibly there is some sub-lethal action involved when spores were exposed at this temperature. This assumption can be substantiated by the work done by Evans and Currans, 1942 (7). They cited early studies on disinfection by Koch, Goffky and Loeffler (1881) in which they reported that anthrax spores which had been heated at 90° C and 95° C required longer to produce visible viable plate colonies than unheated spores.

Since that time the growth delaying action of sublethal heat has been recorded by many investigators, both for spores and vegetative cells. Evans and Currans added that heat as a stimulus to germination has been generally overlooked.

#### The Effect of Lower Temperatures on Spores Suspended in Phosphate Buffered Water

I have set up several experiments to determine if 90° C is too high a temperature. This temperature suggests that it has an inhibiting effect on the growth rate of spores.

Spores were heat treated for 30 minutes at several temperatures (35, 45, 55, 70, 80 and 85° C) in phosphate buffered water. The heated spore samples were subcultured immediately in flasks containing tryptone glucose beef extract broth and incubated at 35° C. Samples were withdrawn from each flask, dispensed in a cuvette and turbidity readings taken. These growth curves at these temperatures were plotted and compared with growth curves on spores treated at 90° C. The data are shown on Figure 7.

Definitely 90° C heat treatment compared to the other temperatures showed a significant effect on slowing the growth rate of the spores. At lower temperatures, the growth rate curves showed minimal difference. However, 55° C appeared to be an optimum temperature. It is quite possible this turbidimetric method is not sensitive enough to measure changes in a germinating spore at the early stages - possibly within the first hour of incubation in broth at 35° C.



### Optical Density Studies

The rate and extent of germination was measured by a decrease in optical density of the spore suspension on a nonheat treated and heat treated spore in phosphate buffered water and subcultured immediately in tryptone glucose beef extract broth at 35° C. The result is shown in Figure 8. The germination rate was essentially the same on both nonheated and heat treated spore suspensions. The extent of germination up to 120 minutes was practically identical.

The effect of the type of suspending medium on the rate and extent of germination is shown in Figure 9. The rate and extent of germination are definitely different on spores treated at the two type of media. It also appears that germination of spores treated at 25° C in tryptone glucose beef extract broth was completed after two hours.

The percentage decrease in optical density as a measure of germination is shown in Table 1. It is obvious from this data that germination was not detected in spores suspended in phosphate buffered water.

It was observed in Figure 10, the effect of 60° C on the minimal decrease in optical density of spores suspended in tryptone glucose beef extract broth. In Figure 11, spores suspended in phosphate buffered water heated at 60° C and subcultured immediately in TGE broth germinated progressively with time. The percentage decrease in optical density is shown in Table 2.

Again in Figure 10 is shown the optical density readings on spores heated in TGE broth suspensions at 60° C. Only a minimal decrease in optical density as a measure of germination is noticeable.

In order to test the overall effect of various temperatures, spores were heat treated at 25, 45, 60, 70, 80, and 90° C in phosphate buffered water for 30 minutes and subcultured immediately in TGE broth. The samples were further incubated at 35° C for 0, 15, 30, 60, and 90 minutes. Figure 12 shows the results of the optical density readings. It appears initially 60° C had an optimum germination effect on spores held at this temperature for 30 minutes, plus a subsequent transfer in TGE broth. The percentage optical density decrease is shown in Figure 12.

Figures 13, 14, 15, and 16 show the added effect on spore germination as subsequent incubation in TGE broth was lengthened from 15, 30, 60, and 90 minutes. The deleterious germination effect is greatest at 90° C. This is very noticeable in Figure 14. It appears that a 90 minutes incubation in TGE broth as shown in Figure 16, the inhibiting effect at 80° C and 90° C was recognizable. The extent of germination is given in percentage loss of optical density as shown in Figures 12, 13, 14, 15, 16, and 17.

A summary of the effect of these various temperatures on these spore suspensions is shown in Figures 17 and Table 3.

It appears in Figure 17 and Table 4 that at 30 minutes incubation at 35° C in TGE broth the nonheat treated spore suspension slightly has a larger loss in optical density.

The results also indicate from all these optical density studies the synergistic effect of spore germination on spores suspended in phosphate buffered water and subsequent treatment of spores in TGE broth.

The effect on spore germination by percentage loss in optical density is shown in Figure 18. Again, the gradual decline in OD loss is seen as the exposure was increased.

#### Packed Cell Volume Increase

Spores suspended in phosphate buffered water at 90° C for 0, 60, 120, and 180 minutes and immediately subcultured in broth for 2 minutes were measured for a volume change. All samples showed an increase of 57%, 77%, and 62% in packed cell volume from the nonheat treated spores. This is shown in Table 4 and Figure 19. Notice the effect on spores suspended in phosphate buffered water 90° C for 0, 60, 120, and 180 minutes. In all cases there is no PCV change.

#### Staining Microscopy

Loss of the primary stain (malachite green) was significantly shown for spores heated at 120 and 180 minutes in Table 4. Only about 5% stainability was lost on spores heated for 60 minutes.

#### Spore Darkening

Spores suspended in phosphate buffered water at 90° C for 60, 120, and 180 minutes showed partial darkening, indicating germination. These results are shown in Table 4.

Figure 20 shows phase contrast photomicrographs of B. subtilis var. niger spores. These spores were nonheat treated and heat treated at various exposure times at 90°C in phosphate buffered water suspension. Microscopic examination revealed partial darkening of the spores in the 30-minute heated sample and progressively increased at longer exposure times. When the viable number of surviving organisms was 190,000 per ml at 120-minute exposure, partial darkening was noticeable on all spores. In a few fields examined, some spores completely darkened, signifying fully germinated spores. Irregular swollen shapes of some spores was noticeable.

Figure 21 (A, B, C) shows electron micrographs (x 20,000) of spores heat treated at 0, 30, and 240 minutes suspended in phosphate buffered water. Figure 21C shows a disintegration of spores as opposed to those shown in Figures 21A and 21B.

#### Summary

The nonlinearity of the survival curves of spores of B. subtilis var. niger shown in Figure 1 is attributed to germination events along the induction period and a rapid decline in spore numbers with increase in exposure times. Although most investigators subscribe to this nonlinearity of survival curves under moist conditions, very little data are available as to the mechanism of thermal inactivation under these conditions.

I have illustrated the effective destruction of spores suspended in a nutrient medium as opposed to spores suspended in phosphate buffered water. Failure to detect any optical density decrease as a measure of germination on spores heat treated in phosphate buffered water does not necessarily mean non-metabolic activity in that spore. In fact, when heat treated spores suspended in phosphate buffered water were subcultured immediately in a nutrient medium, germination was initiated, indicating a synergistic effect. This observation is consistent with the findings shown by other investigators. Frequently, a known enzyme, or enzymes such as L-alanine, adenosine, and others are used in germination studies. When heat was applied together with an enzyme in the medium, spore germination was triggered in a matter of seconds or several minutes, depending upon the species or strain.

In another study where staining and packed-cell volume was determined, a nutrient medium was needed to detect changes in stainability or increase in spore volume.

In phase-contrast microscopy, at least partial darkening was evident on heat treated spores. The change from refractility to nonrefractility would have been magnified if heat treated spores suspended in buffered water were immediately transferred in nutrient broth and viewed under phase microscopy.

#### Scanning Electron Microscopy.

Scanning electron microscopy was used to monitor events based on morphological changes on nonheat treated and heat treated spores. It appeared that severe heating in phosphate buffered water caused a collapse of the germinated spores.

## Appendix

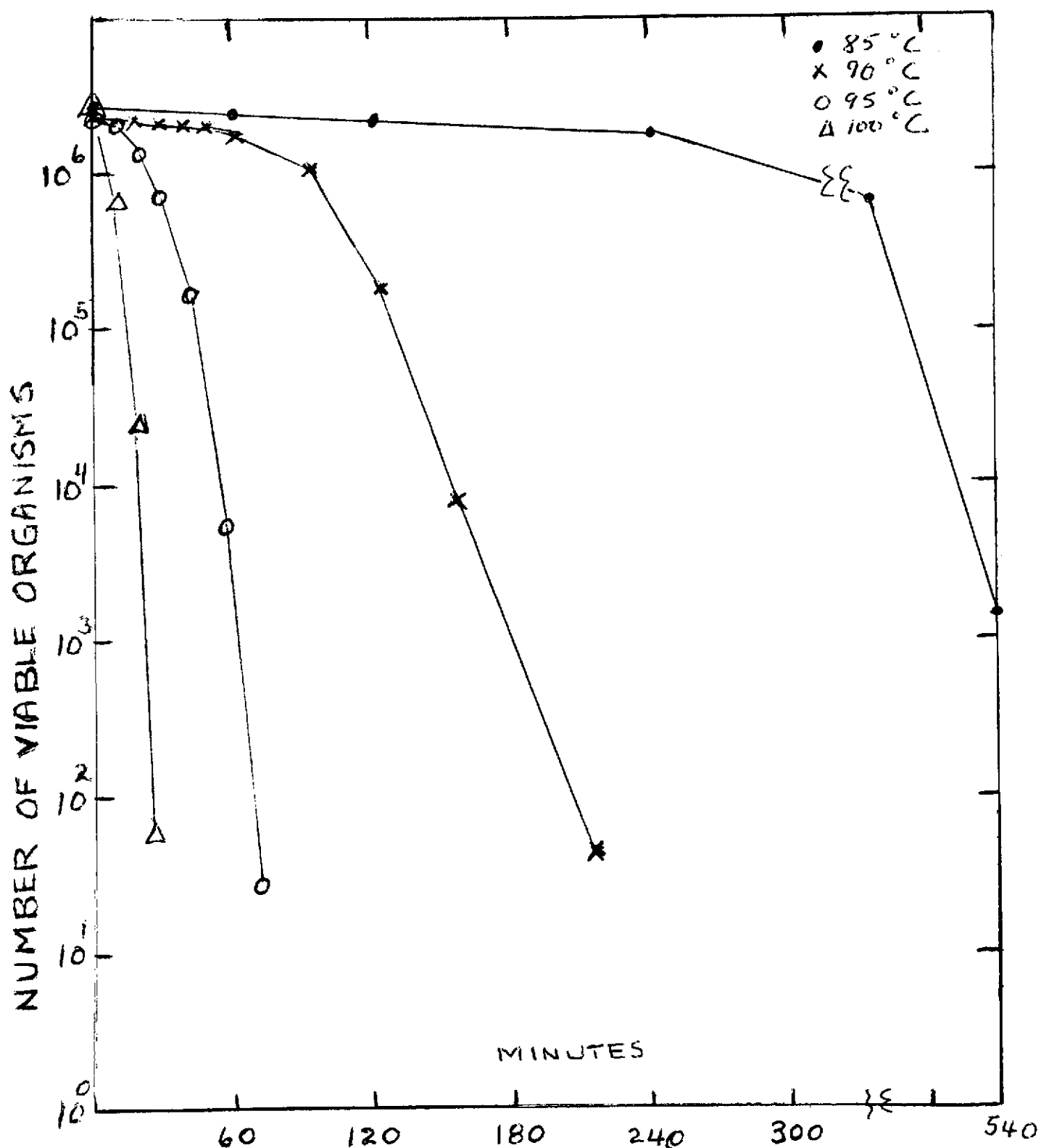


FIGURE 1 - HEAT INACTIVATION OF *BACILLUS* SPORES AT 85°C, 90°C, 95°C AND 100°C IN PHOSPHATE BUFFERED WATER

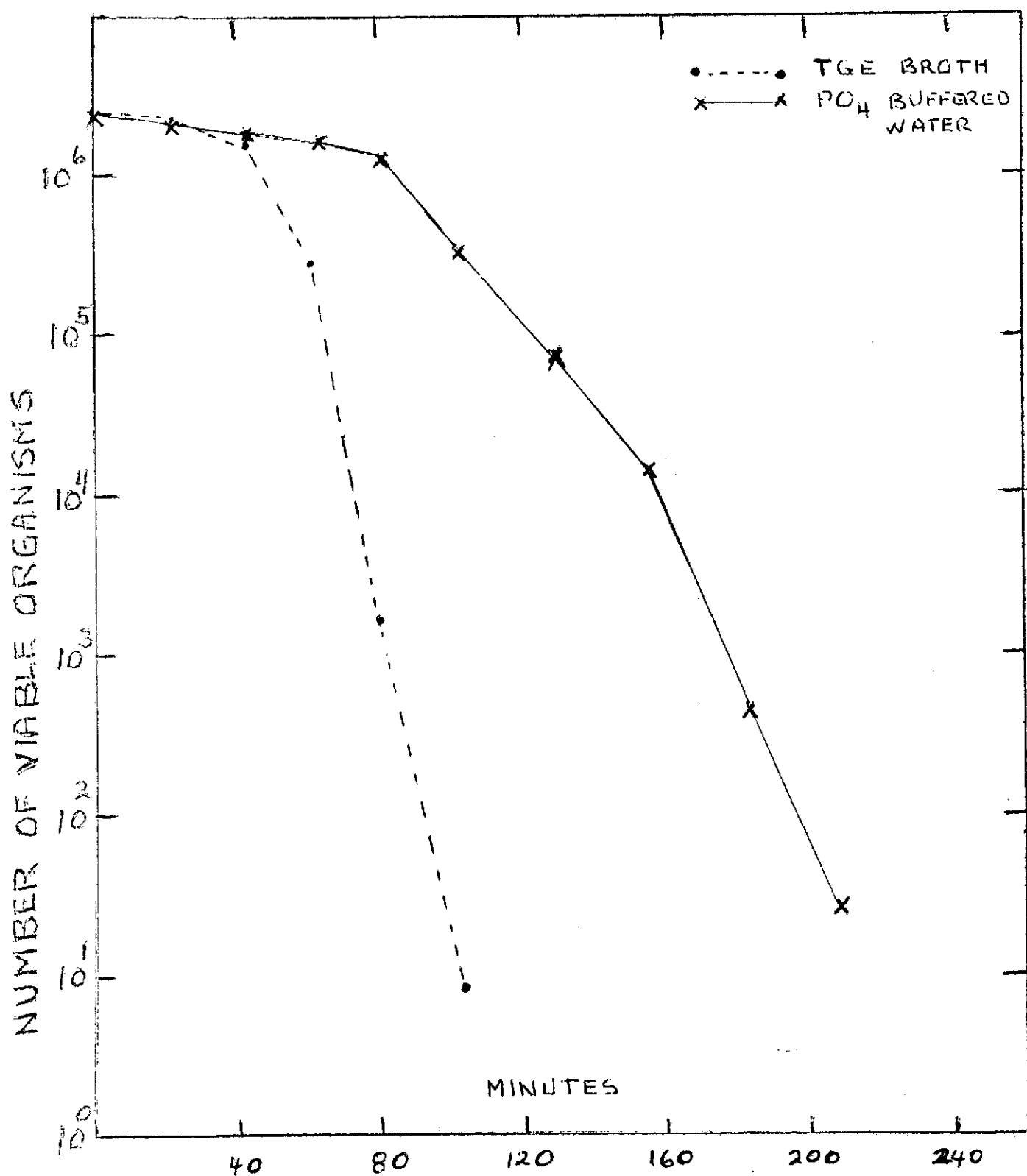


FIGURE 2 - THE EFFECT OF SUSPENDING MEDIUM ON THE THERMAL INACTIVATION OF BACILLUS SUBTILIS VAR. NIGER SPORES AT 90°C



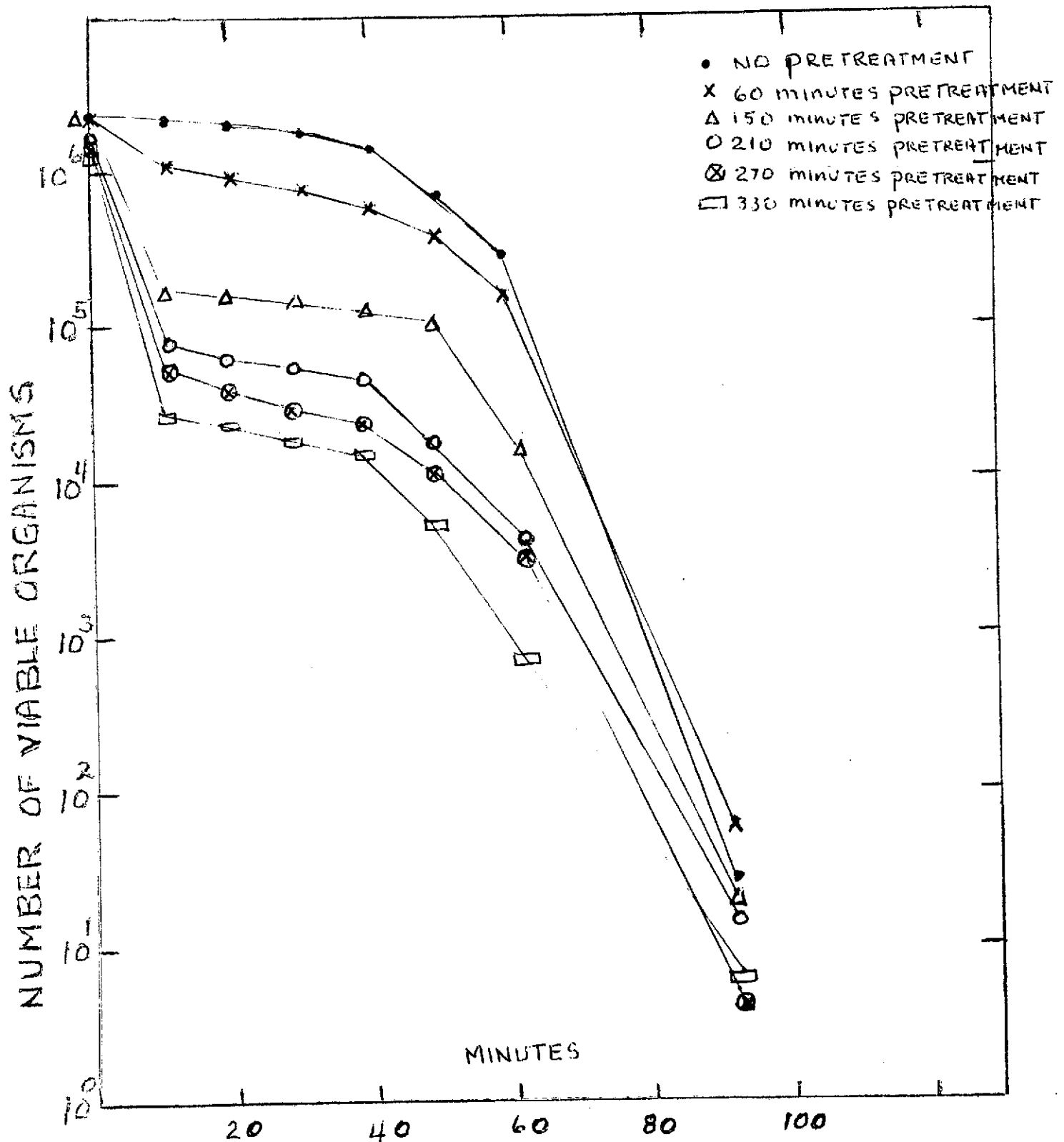


FIGURE 3. THE EFFECT OF 77°F PRETREATMENT IN TGE BROTH PLUS 90% ON THE INACTIVATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES

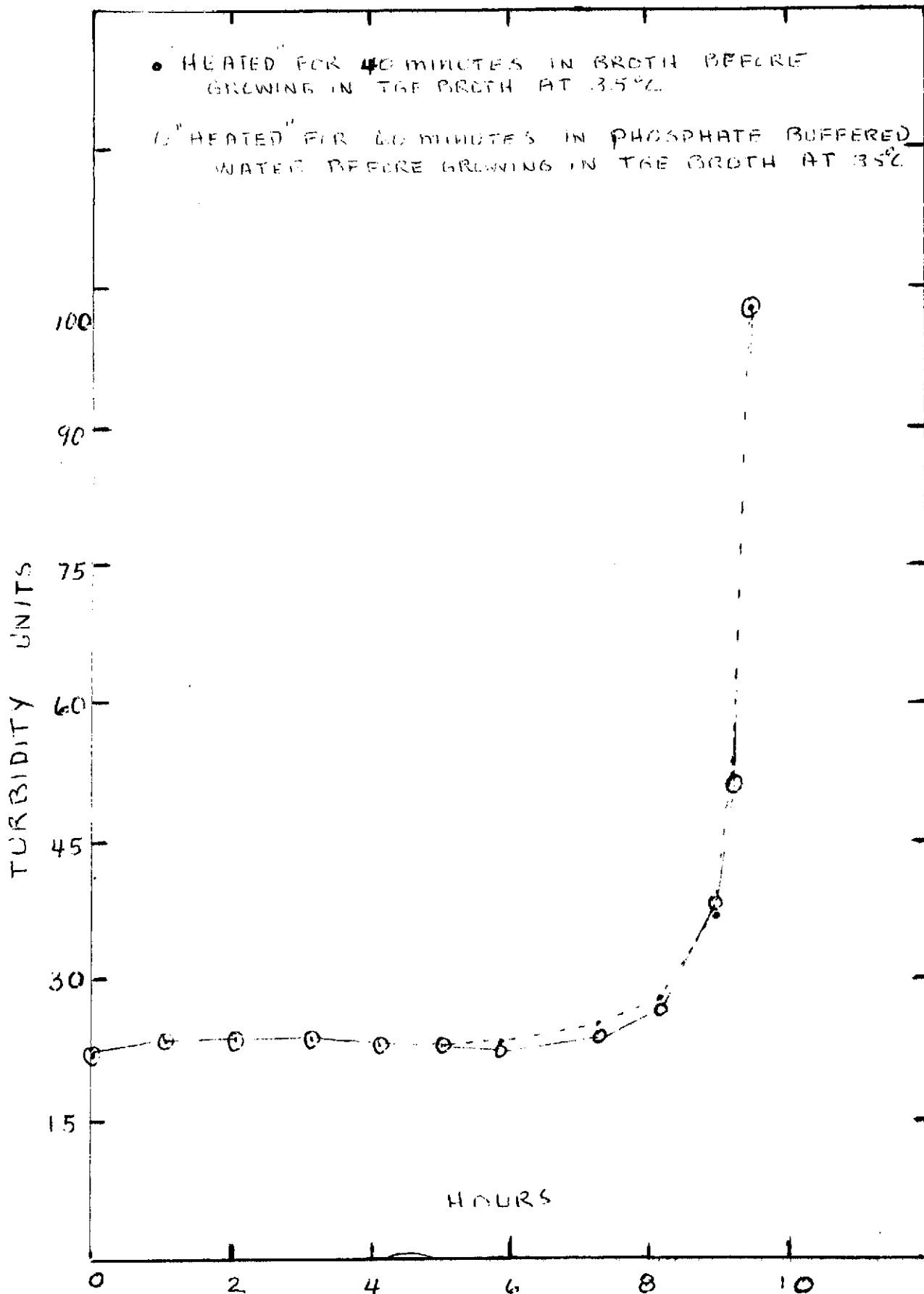
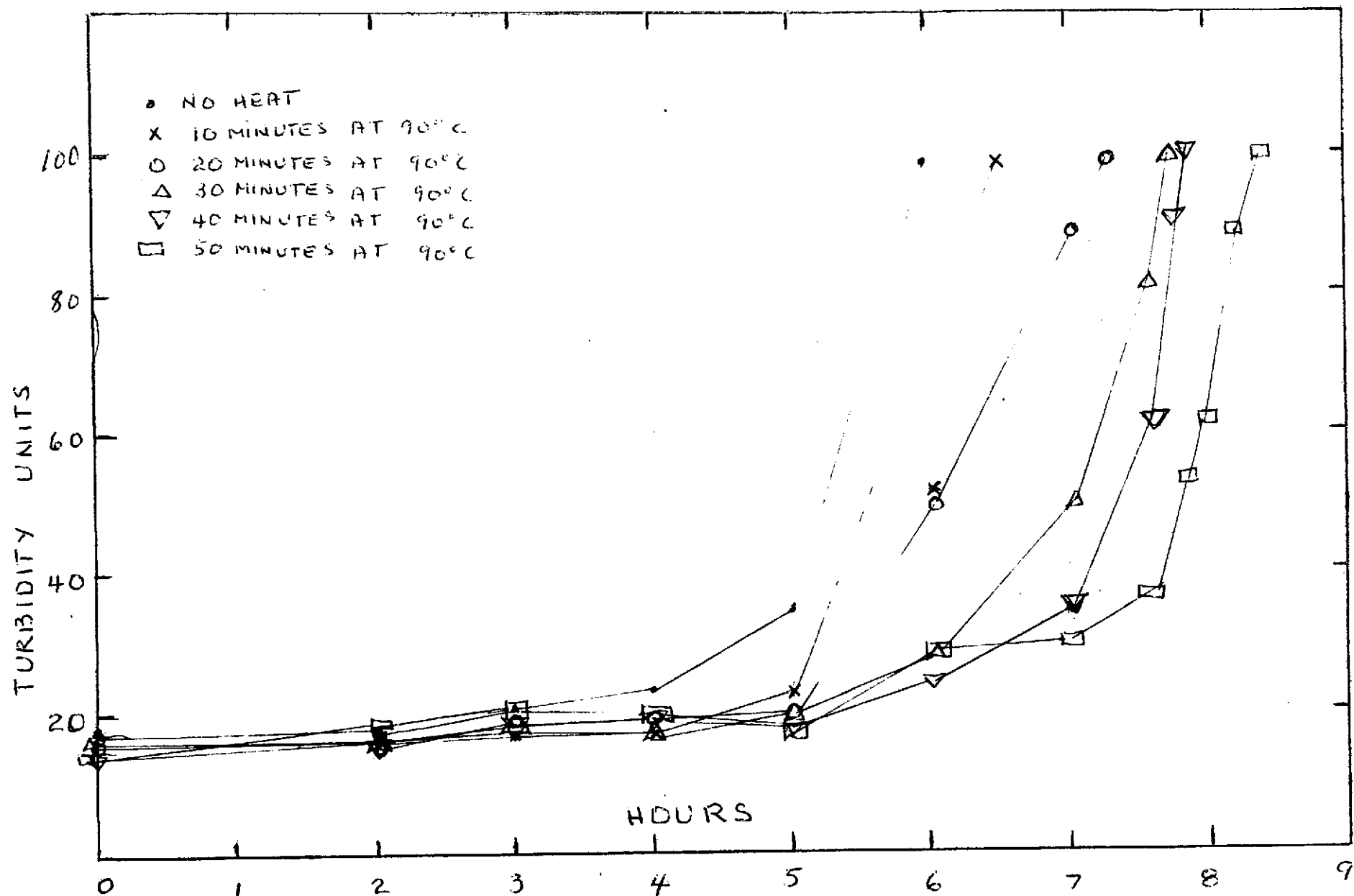
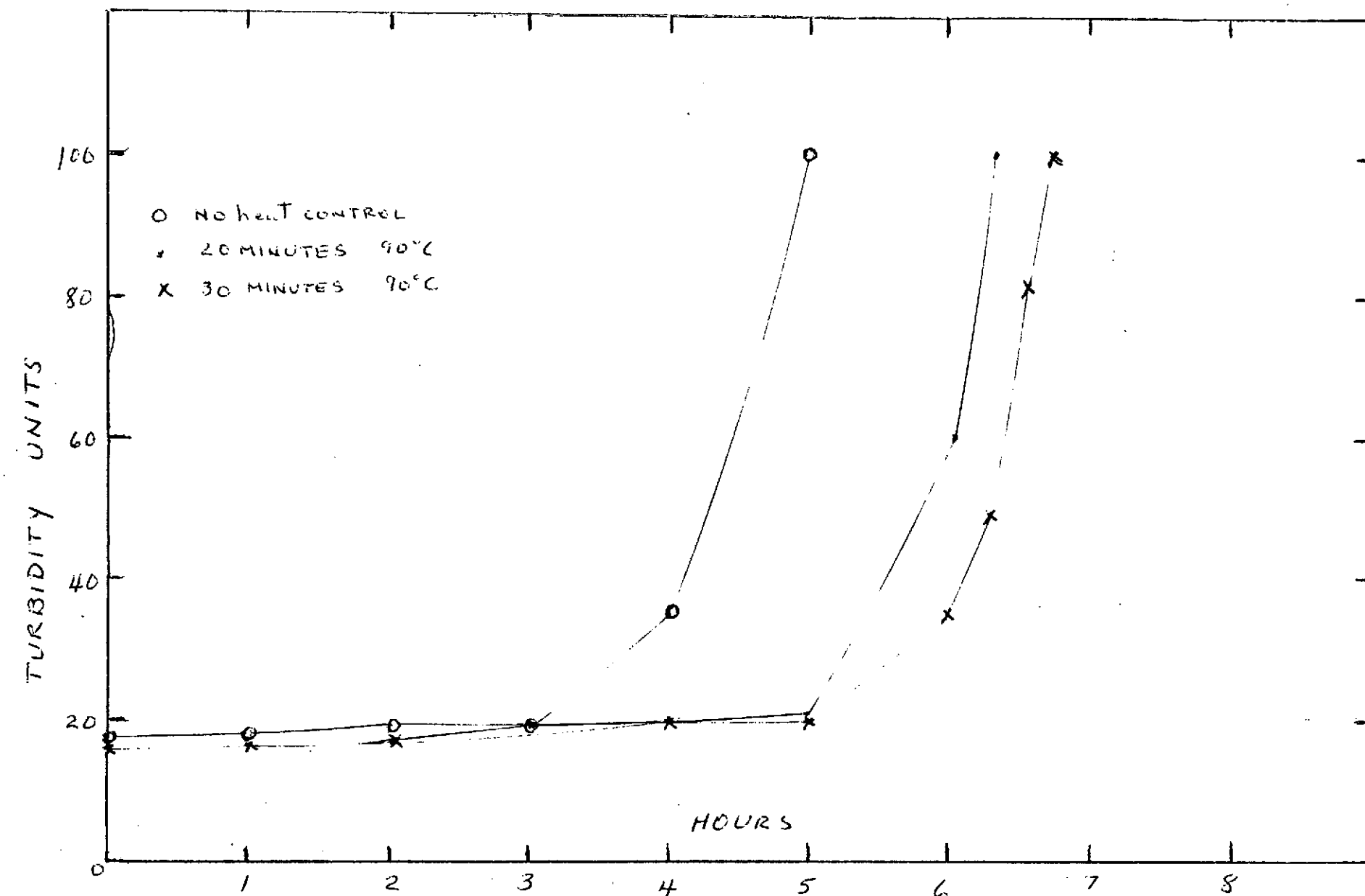


FIGURE 4 - THE EFFECT OF 90°C ON THE GROWTH RATE OF BACILLUS SUBTILIS VAR. NIGER SPORES



GROWTH CURVES OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES HEATED  
IN PHOSPHATE BUFFERED WATER AT 90°C AND SUBCULTURED  
IN TGE BROTH AT 35°C  
FIGURE 5



GROWTH CURVES OF BACILLUS SUBTILIS VAR. NIGER SPORES HEATED IN PHOSPHATE BUFFERED WATER AT 90°C AND SUBCULTURED IN TGE BROTH AT 35°C

FIGURE 6

5-16-55

GROWTH CURVES OF *BACILLUS SUBTILIS*  
VAR. NIGER SPORES HEATED AT  
8 DIFFERENT TEMPERATURES FOR  
30 MINUTES IN PHOSPHATE BUFFERED  
DILUTION WATER AND SUBCULTURED  
IN TGE BROTH AT 35°C

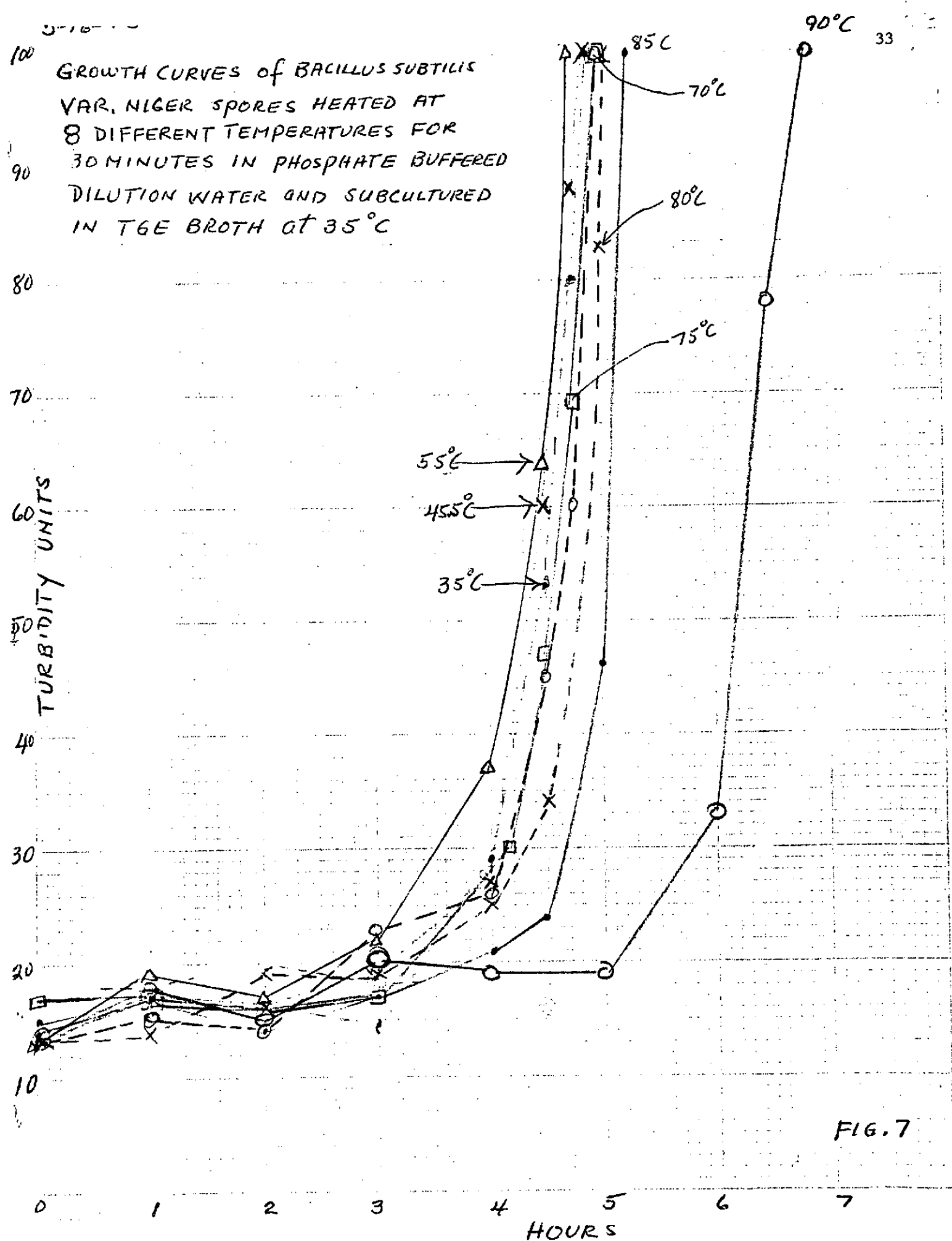
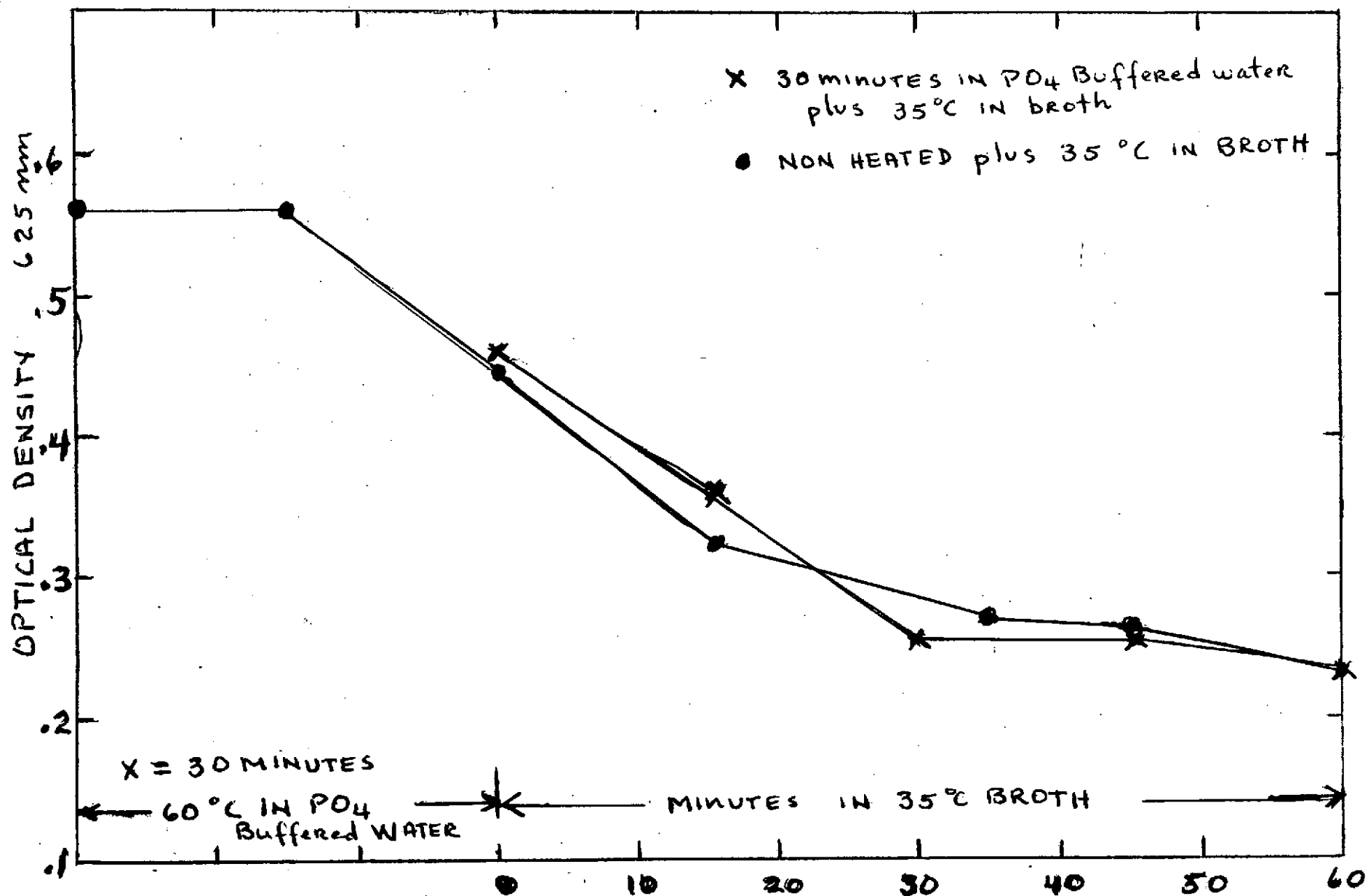
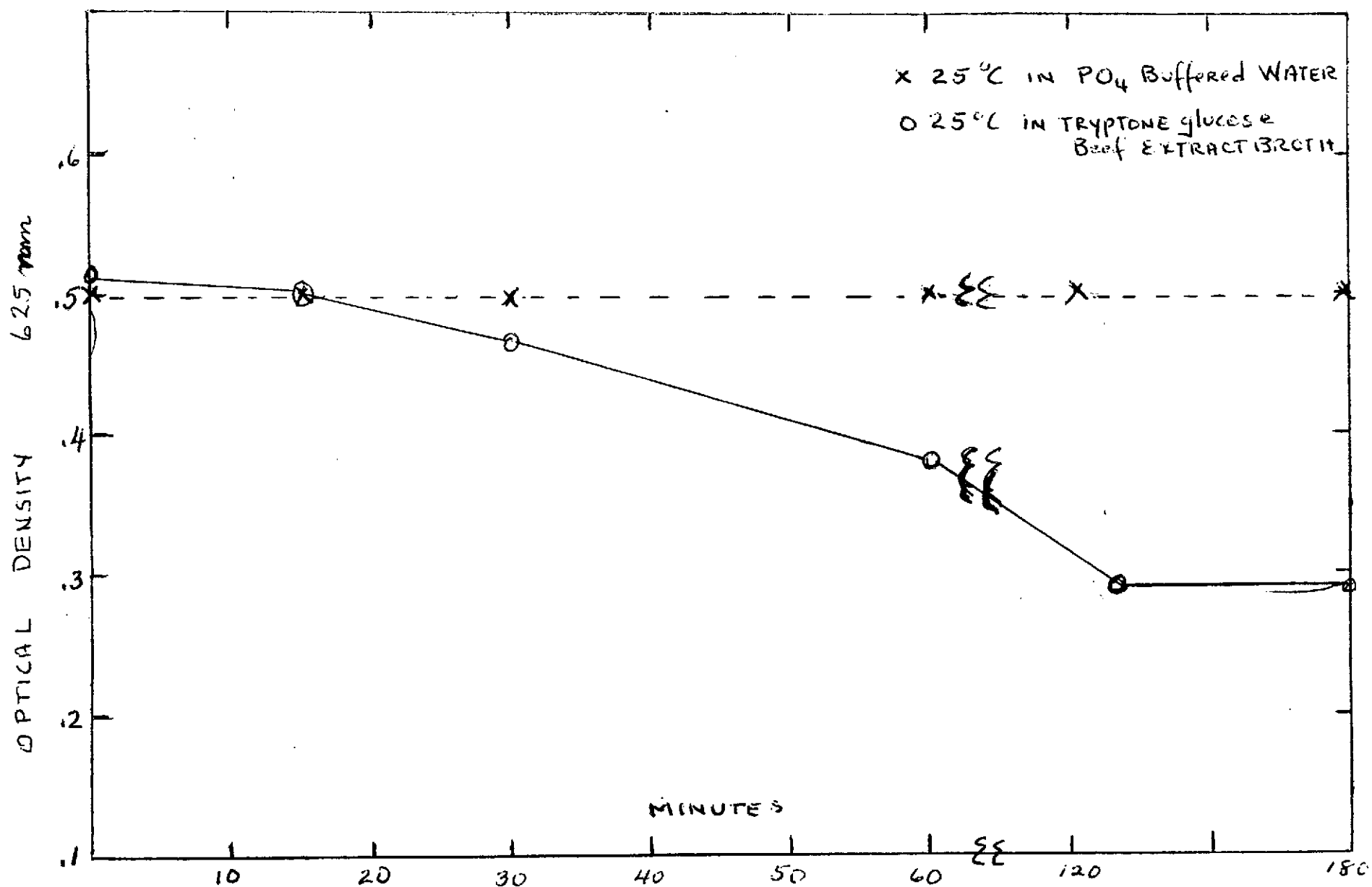


FIG. 7



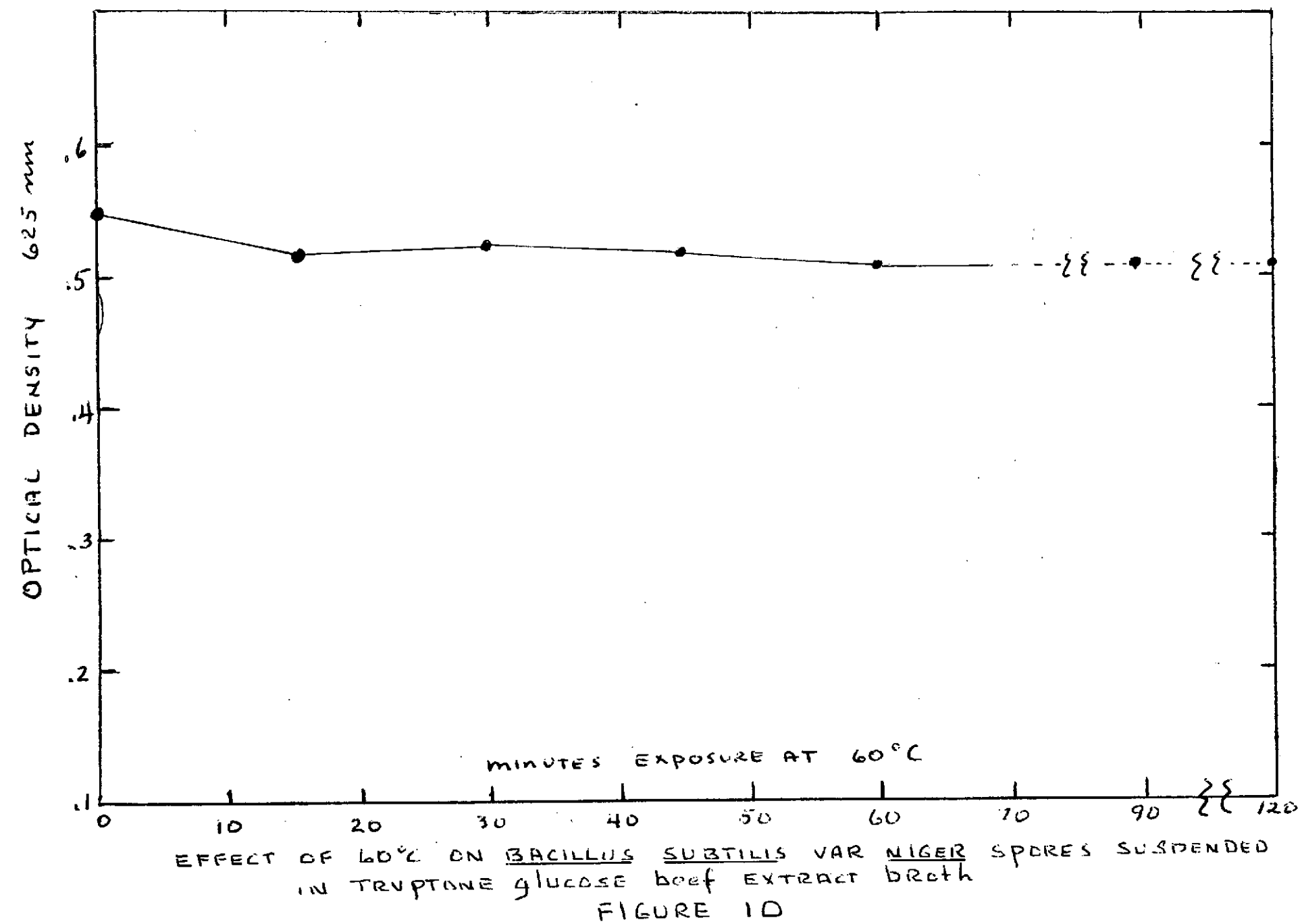
EFFECT OF NON HEATED AND HEATED SPORES IN  $PO_4$  BUFFERED WATER AND SUBCULTURED IMMEDIATELY IN TRYPTONE GLUCOSE BEEF EXTRACT BROTH AT  $35^\circ C$

FIGURE 8

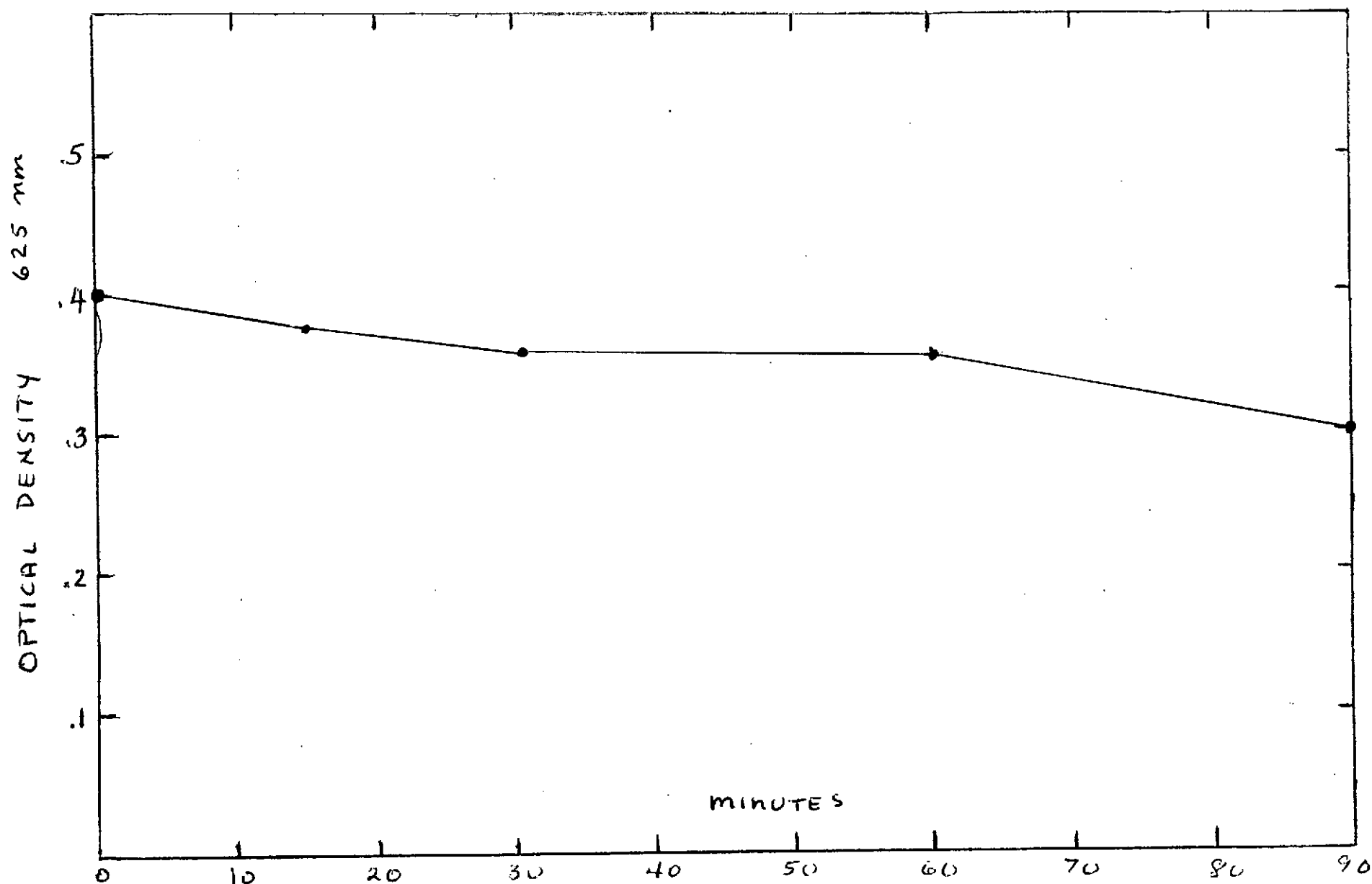


EFFECT OF SUSPENDING MEDIUM ON THE RATE AND EXTENT OF GERMINATION  
ON BACILLUS SUBTILIS VAR NIGER SPORES

FIGURE 9

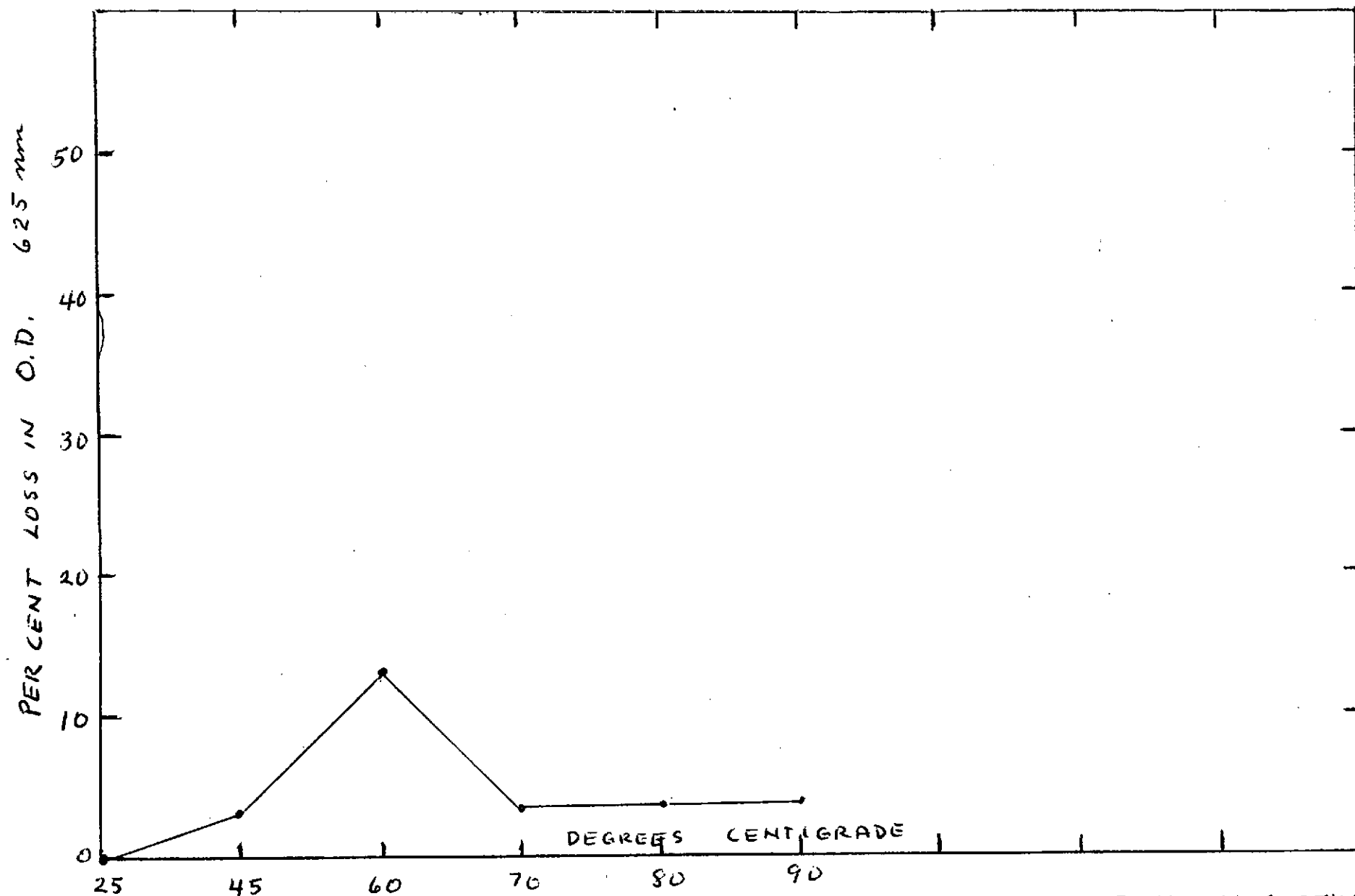






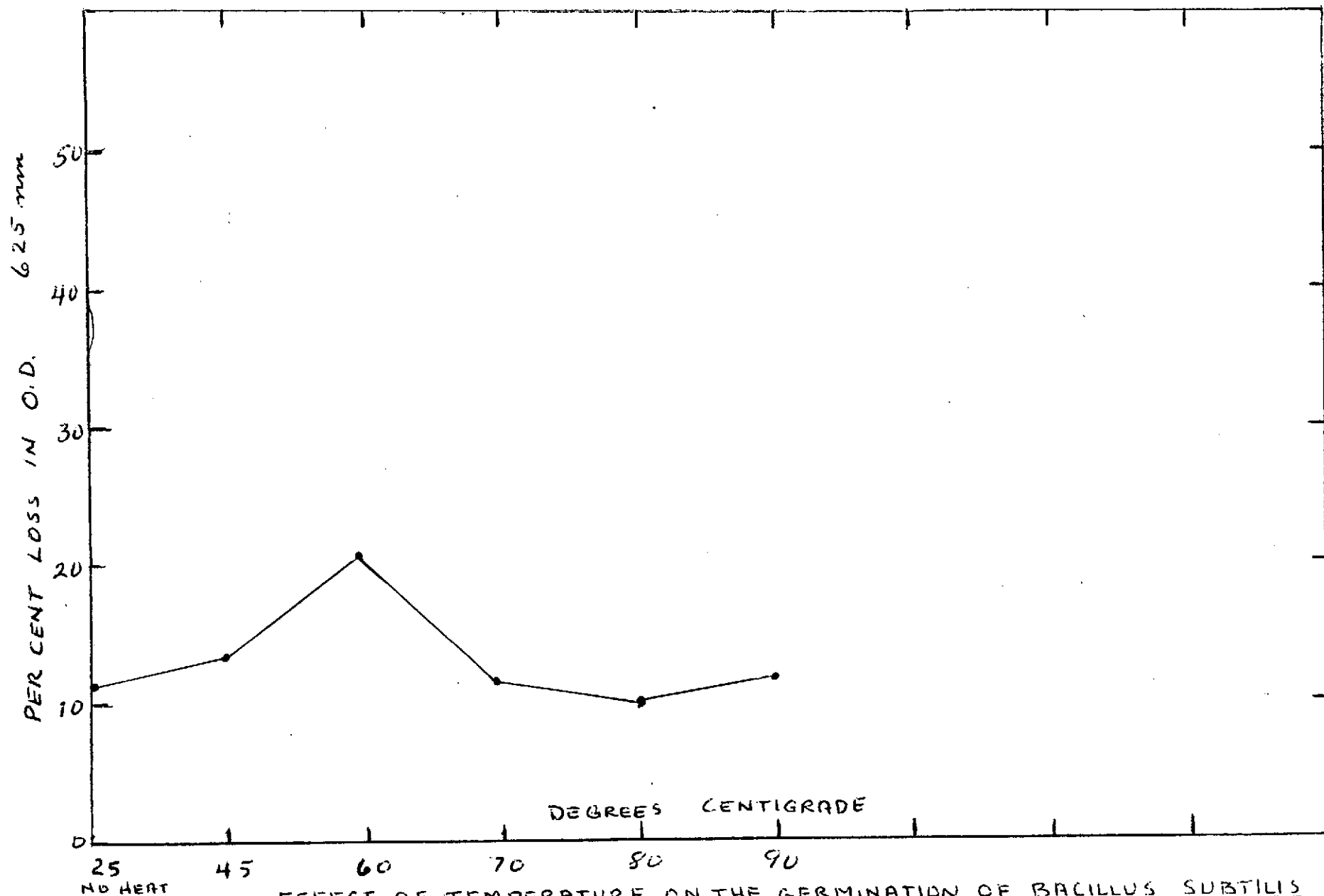
EFFECT OF 60°C ON *BACILLUS SUBTILIS* VAR *NIGER* SPORE SUSPENDED  
IN PHOSPHATE BUFFERED WATER AND IMMEDIATELY SUBCULTURED  
IN TRYPTONE GLUCOSE BEEF EXTRACT BROTH

FIGURE 11



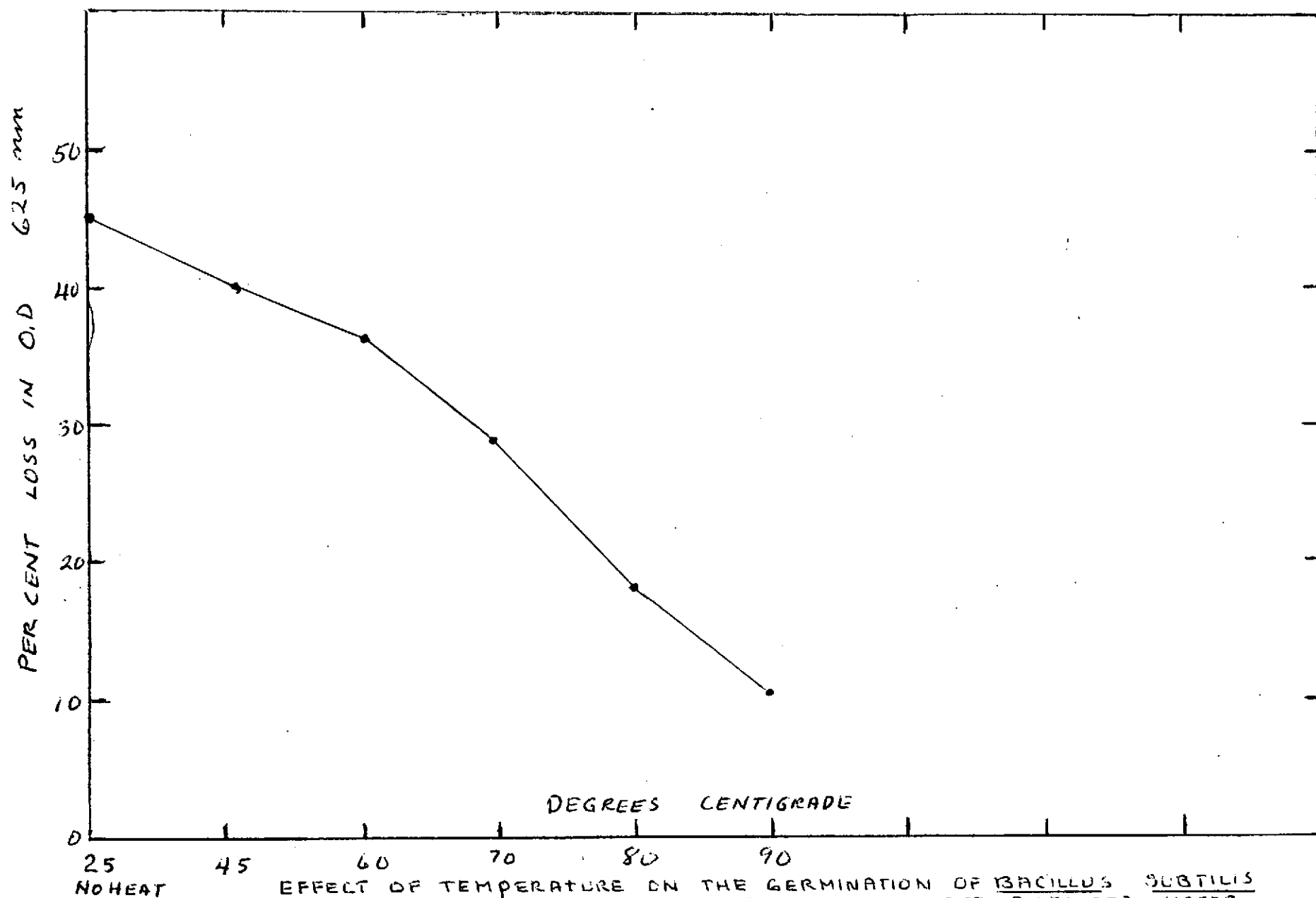
EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES SUSPENDED IN PHOSPHATE BUFFERED WATER FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN TGE BROTH AT 35°C FOR 0 MINUTES

FIGURE 12



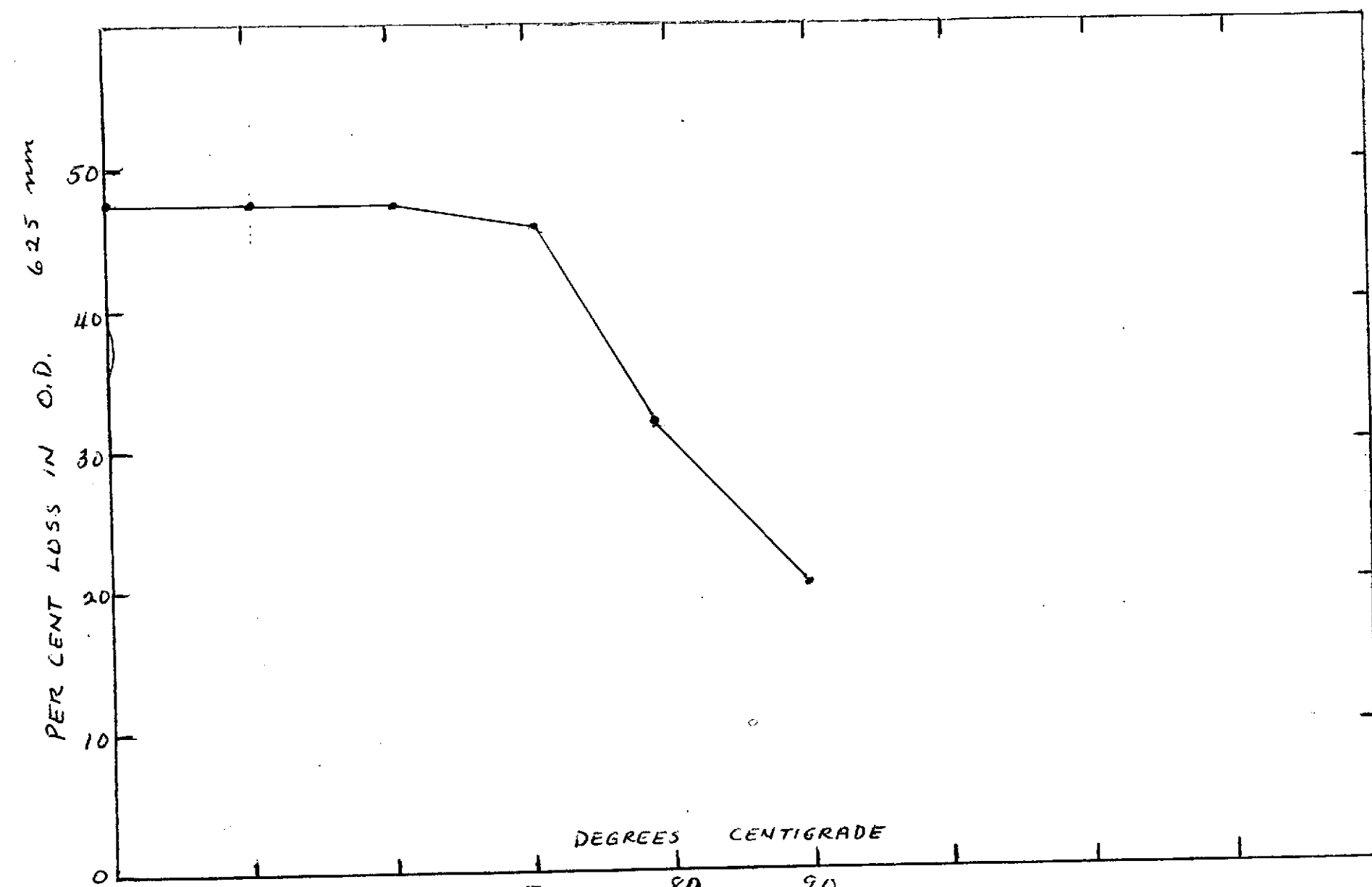
EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS*  
 VAR. *NIGER* SPORES SUSPENDED IN PHOSPHATE BUFFERED WATER  
 FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN TGE  
 BROTH AT 35°C FOR 15 MINUTES

FIGURE 13



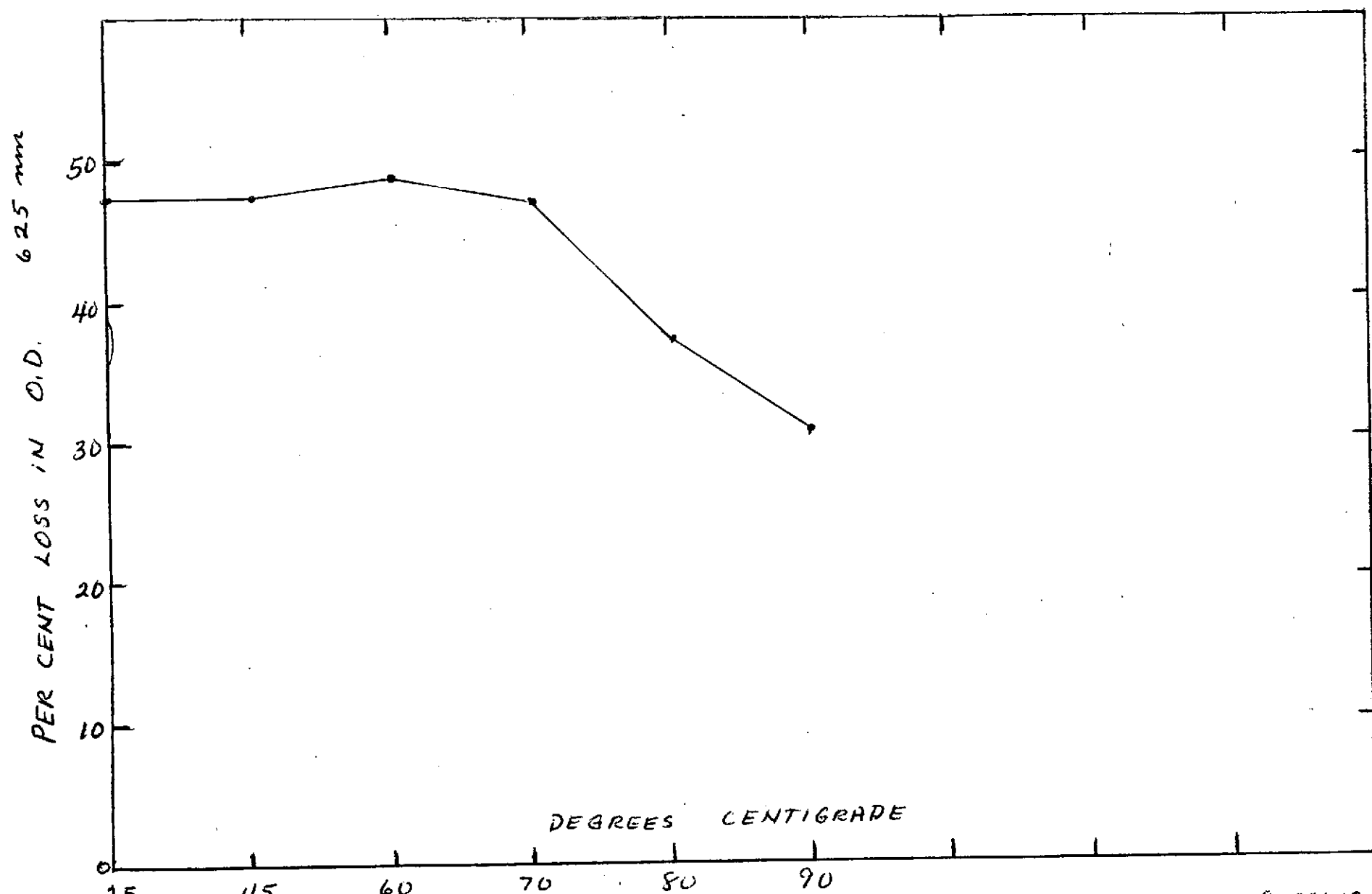
EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES SUSPENDED IN PHOSPHATE BUFFERED WATER FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN TGE BROTH AT 35°C FOR 30 MINUTES

FIGURE 14



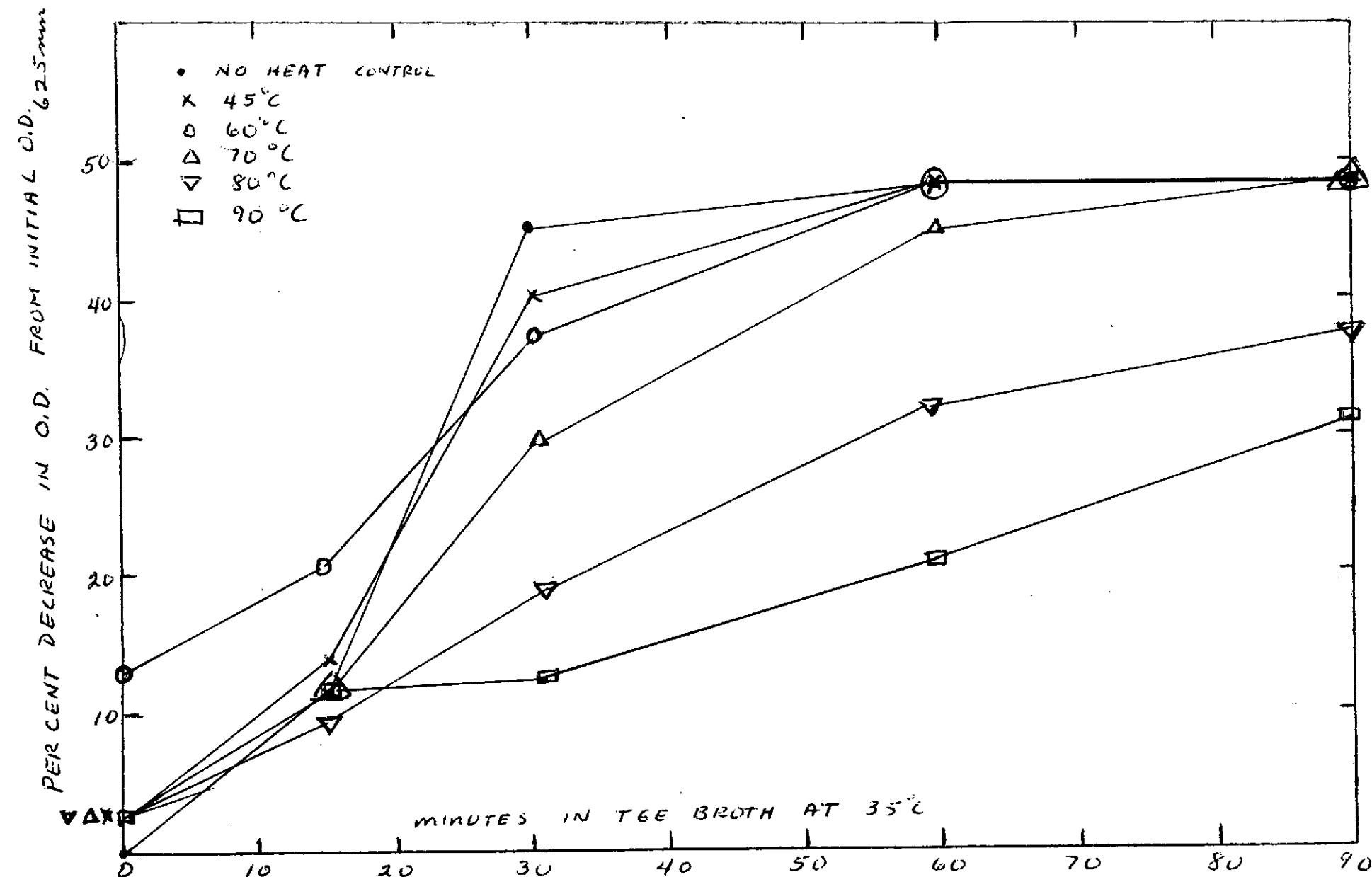
EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES SUSPENDED IN PHOSPHATE BUFFERED WATER FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN TGE BROTH AT 35°C FOR 60 MINUTES

FIGURE 15



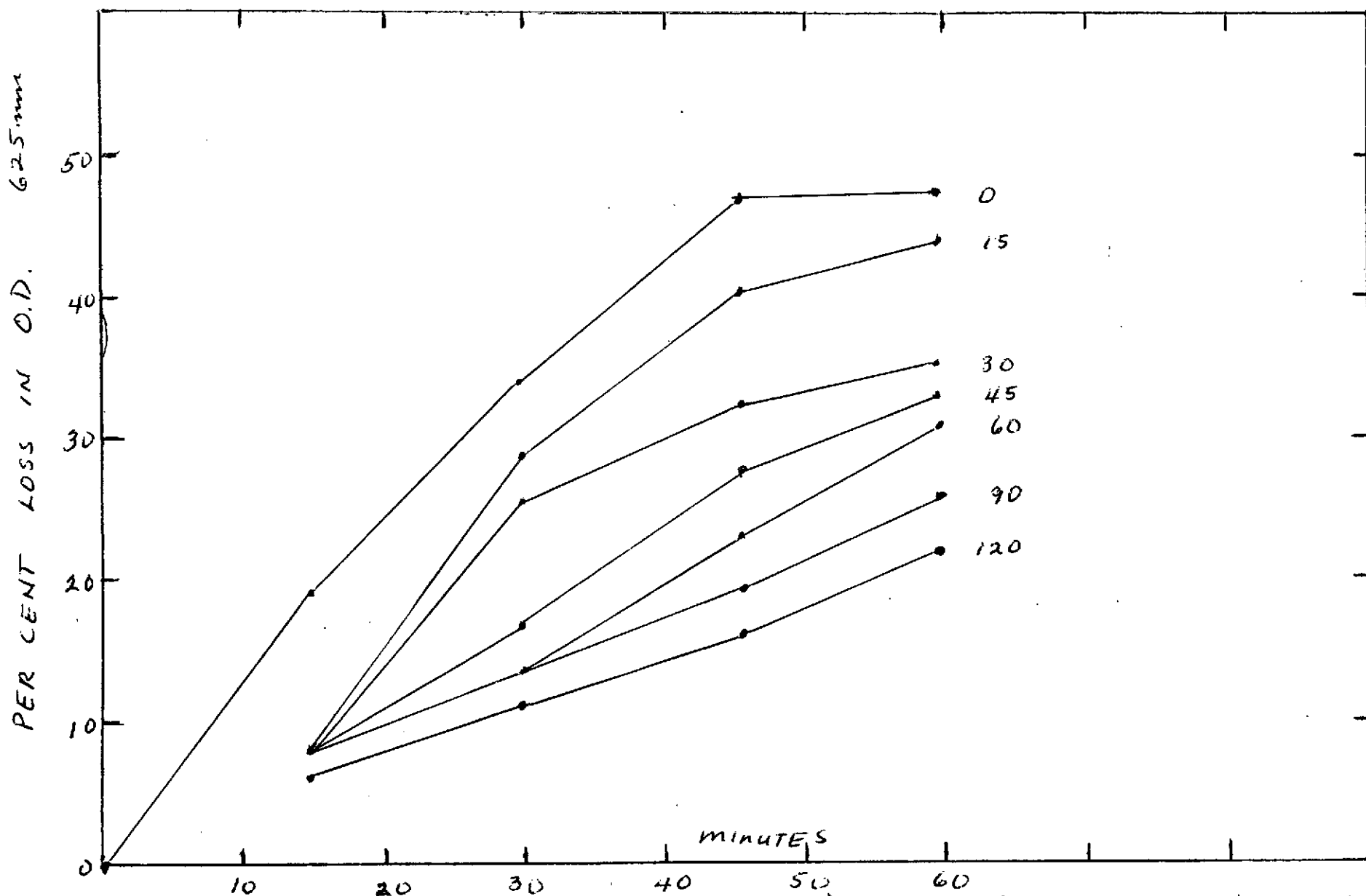
EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS*  
 VAR. *NIGER* SPORES SUSPENDED IN PHOSPHATE BUFFERED WATER  
 FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN T6E  
 BROTH AT 35°C FOR 90 MINUTES

FIGURE 16



EFFECT OF TEMPERATURE ON THE GERMINATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES HEATED IN PHOSPHATE BUFFERED WATER FOR 30 MINUTES AND IMMEDIATELY SUBCULTURED IN TGE BROTH AT 35°C

FIGURE 17



EFFECT OF SUSPENDING MEDIUM (TGE BROTH) ON THE GERMINATION OF *BACILLUS SUBTILIS* VAR *NIGER* SPORES EXPOSED AT 60°C FOR 0, 15, 30, 45, 60, 90 AND 120 MINUTES AND SUBSEQUENTLY INCUBATED AT 35°C

FIGURE 18



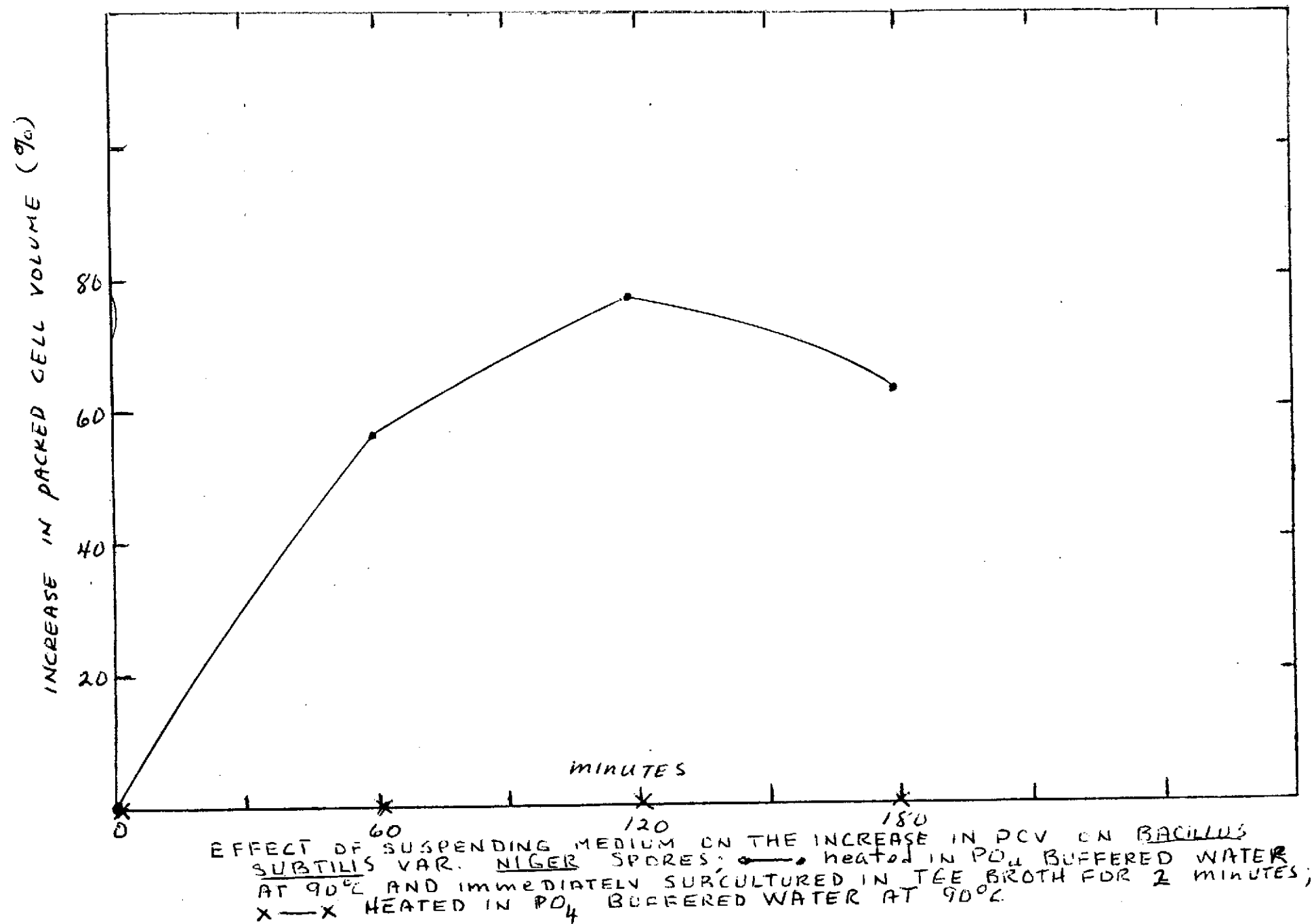


FIGURE 19

Table 1. Effect of suspending medium on the extent of germination of Bacillus subtilis var. niger spores

Exposure at 25° C (minutes)	% optical density decrease as a measure of germination	
	PO <sub>4</sub> buffered water	TGE broth
0	0	0.0
15	0	3.8
30	0	11.5
60	0	30.8
130	0	48.1
180	0	48.1

Table 2. Effect of 60° C on the germination of Bacillus subtilis var. niger spores heated in phosphate buffered water and subcultured immediately in TGE broth at 35° C

60° C in minutes	% O.D. loss
Control No heat treatment	0
15	7.5
30	15.0
60	15.0
90	25.0

Table 3. Effect of temperature on the germination of Bacillus subtilis var. niger spores heated in phosphate buffered water for 30 minutes and subcultured immediately in TGE broth at 35° C

Exposure 30 minutes	% optical density decrease as a measure of extent of germination after incubation in TGEB at 35° C (minutes)				
	0	15	30	60	90
No heat treatment	0	11.1	44.4	46.7	46.7
45° C	2.2	13.3	40.0	46.7	46.7
60° C	13.3	20.0	35.6	46.7	48.9
70° C	2.2	11.1	28.9	44.4	46.7
80° C	2.2	8.9	17.8	31.1	37.8
90° C	2.2	11.1	11.1	20.0	31.1

Table 4. Effect of 90 C on Bacillus subtilis var. niger  
spores suspended in phosphate buffered water

Method	No heat treatment	1 hour 90 C	2 hours 90 C	3 hours 90 C
Heat sensitivity	$2.3 \times 10^6$	$1.8 \times 10^6$	$1.9 \times 10^5$	$8.7 \times 10^3$ <sup>b</sup>
Phase darkening	Refractile	Partial darkening	Partial darkening	Partial darkening
O.D. loss	-- <sup>c</sup>	--	--	--
Stainability loss <sup>d</sup>	0	5%	> 95%	99%
Packed cell volume <sup>d</sup> increase	100	157%	177%	162%

<sup>a</sup> Based on number of viable organisms/ml by plate count method.

<sup>b</sup> At 2-1/2 hours.

<sup>c</sup> No data.

<sup>d</sup> Immediately subcultured in broth for 2 minutes.

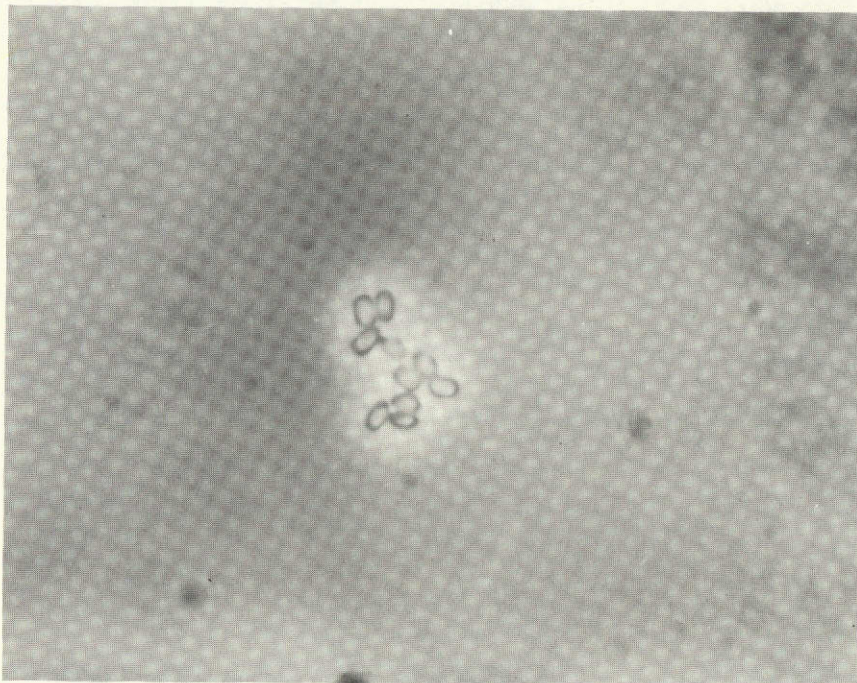


Figure 20a. No heat control. Spores suspended in phosphate buffered water.

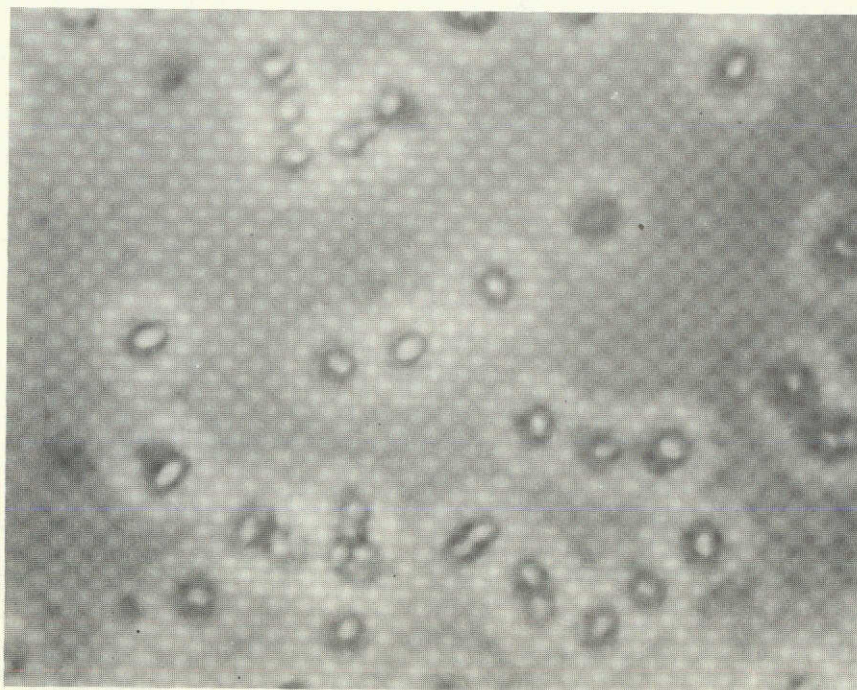


Figure 20b. Spores suspended in phosphate buffered water and heated at 90° C for 15 minutes.,



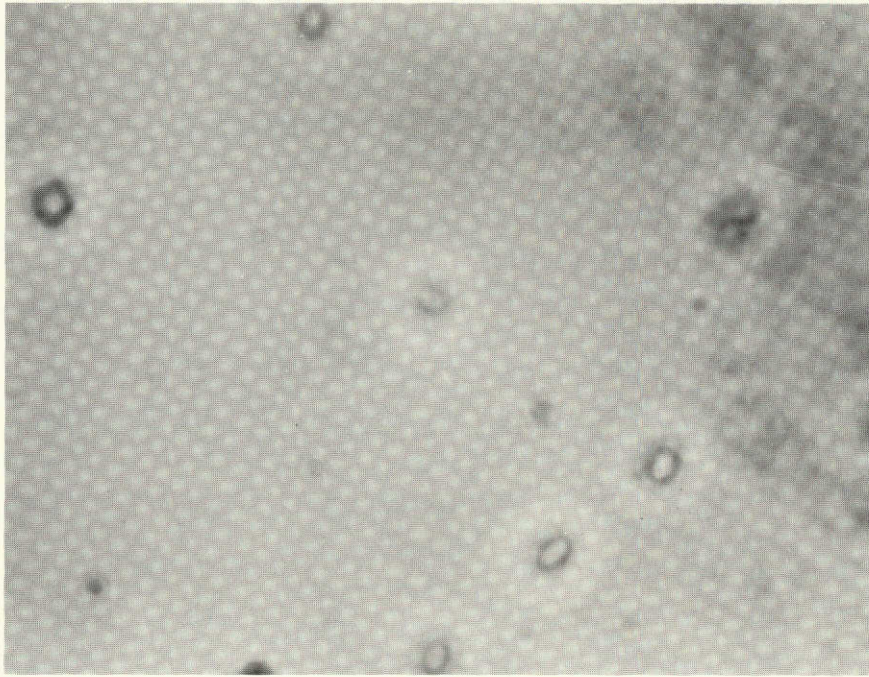


Figure 20c. Spores suspended in phosphate buffered water and heated at 90° C for 30 minutes.

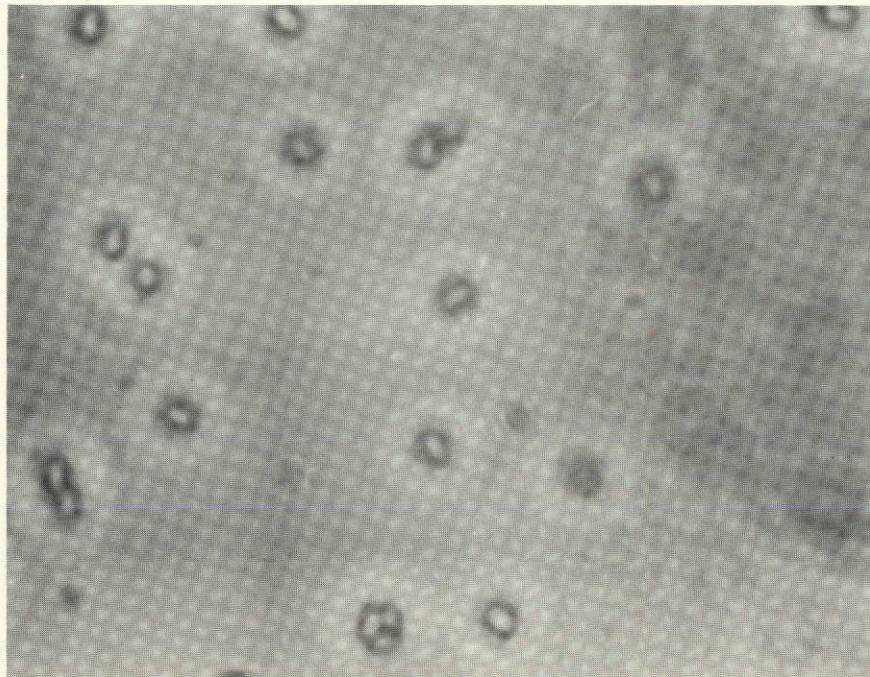


Figure 20d. Spores suspended in phosphate buffered water and heated at 90° C for 45 minutes.



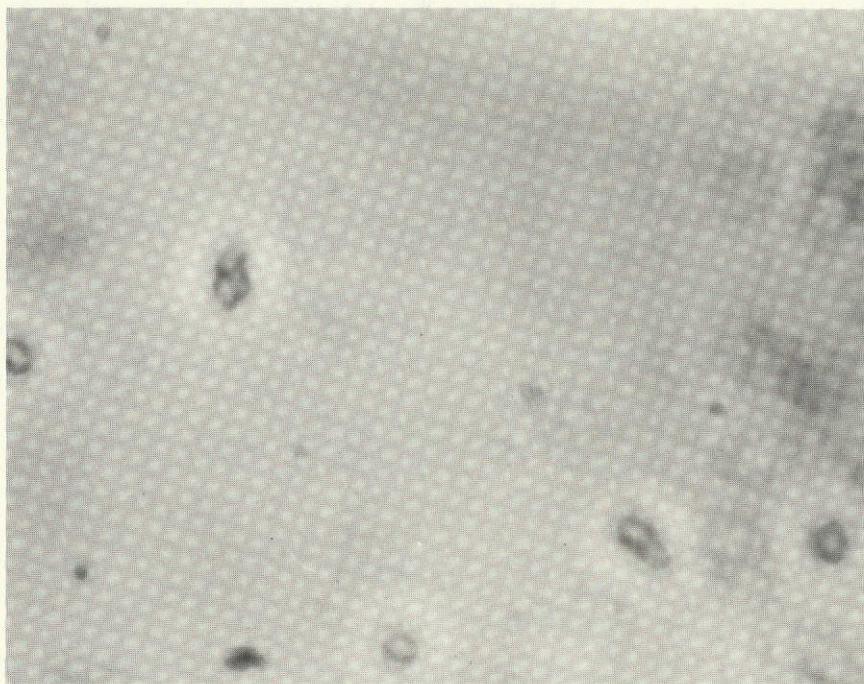


Figure 20e. Spores suspended in phosphate buffered water and heated at 90°C for 60 minutes.

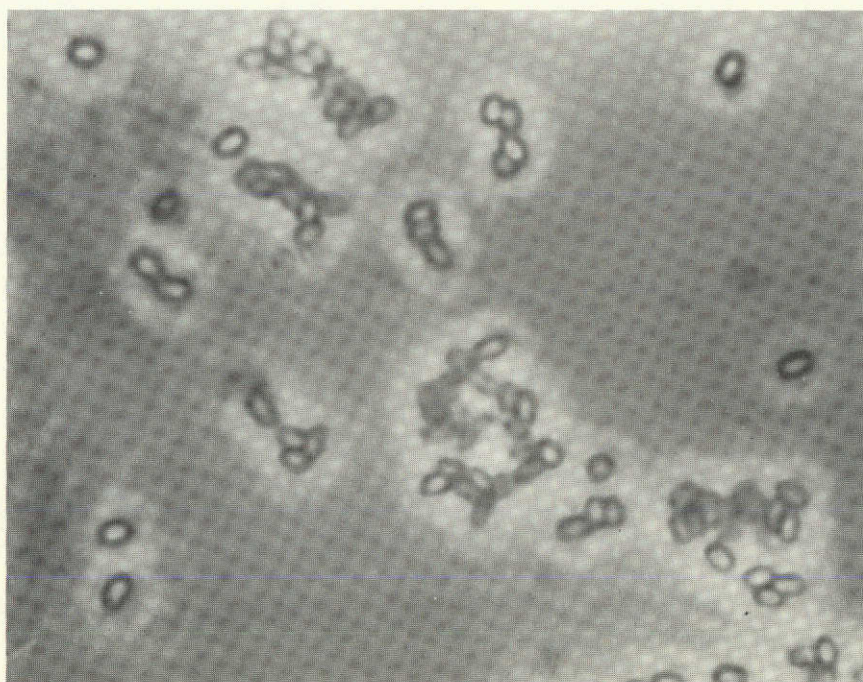


Figure 20f. Spores suspended in phosphate buffered water and heated at 90° C for 75 minutes.



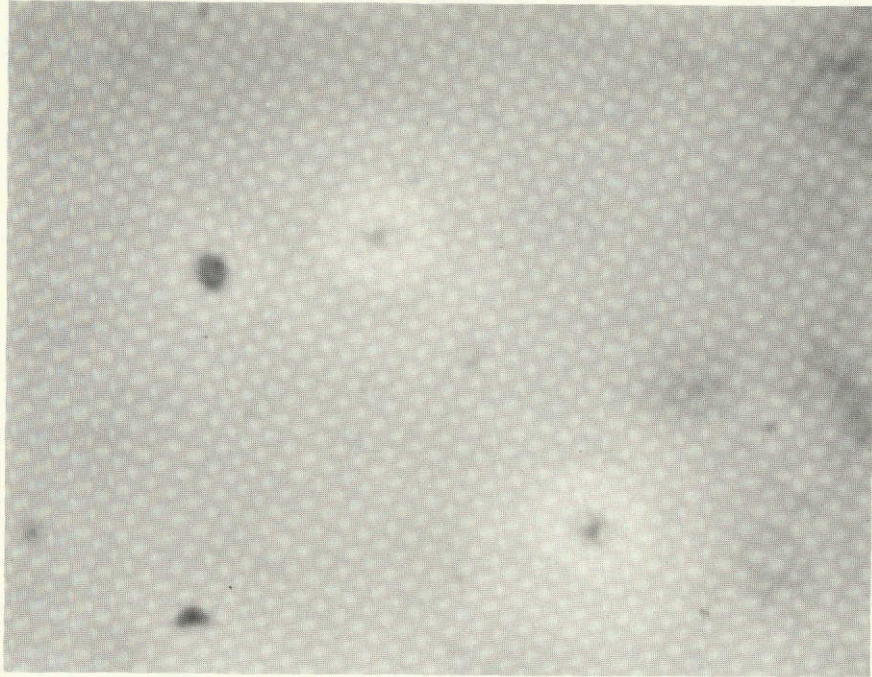


Figure 20g. Spores suspended in phosphate buffered water and heated at 90° C for 120 minutes.

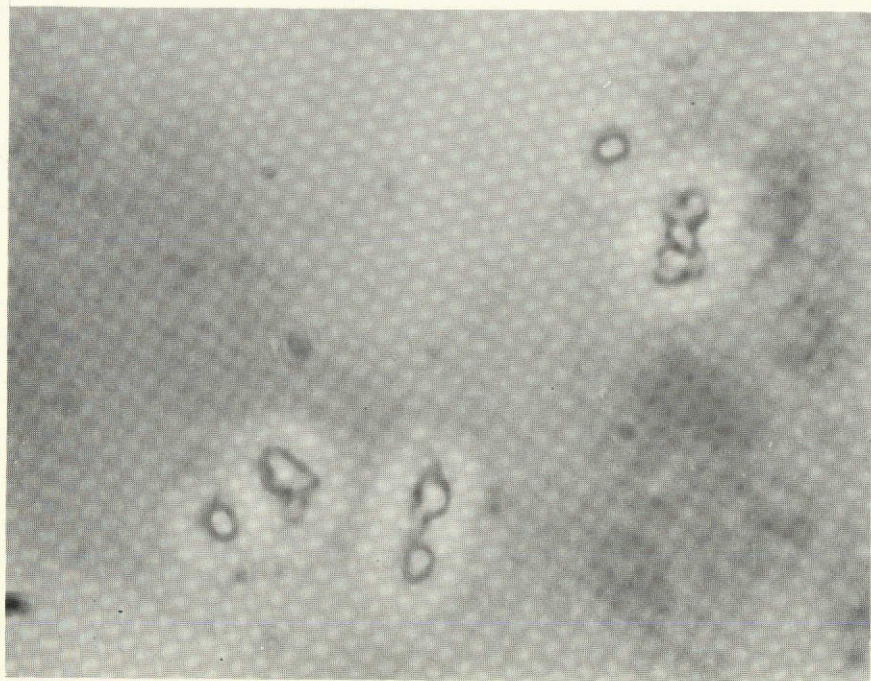


Figure 20h. Spores suspended in phosphate buffered water and heated at 90° C for 120 minutes.

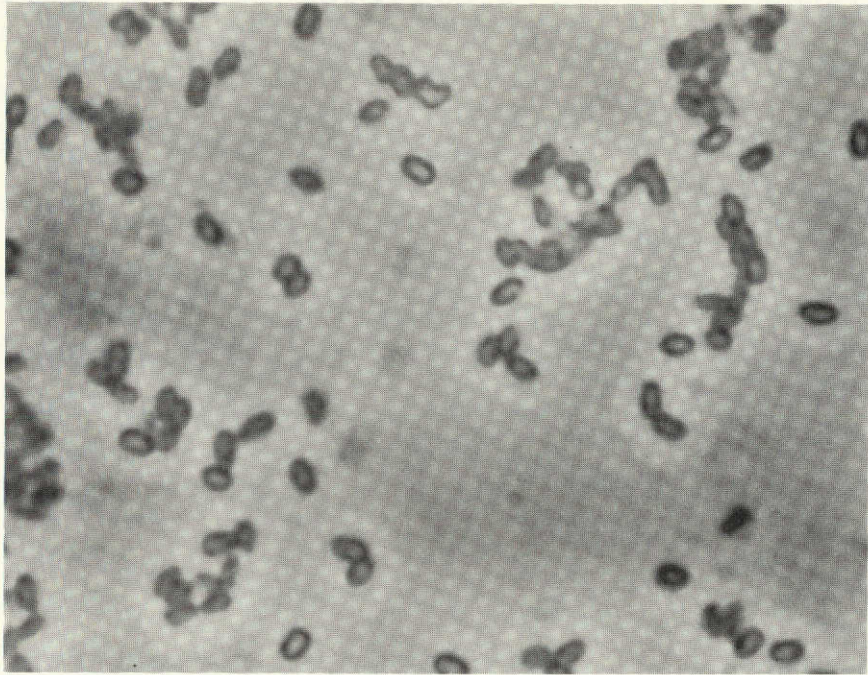


Figure 20i. Spores suspended in phosphate buffered water and heated at 90° C for 180 minutes.



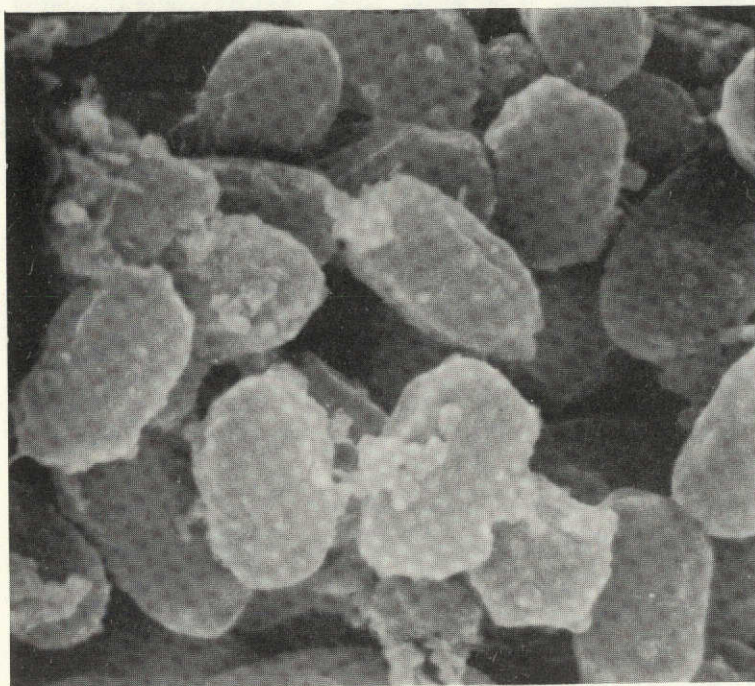


Figure 21a. Bacillus subtilis var. niger  
spores

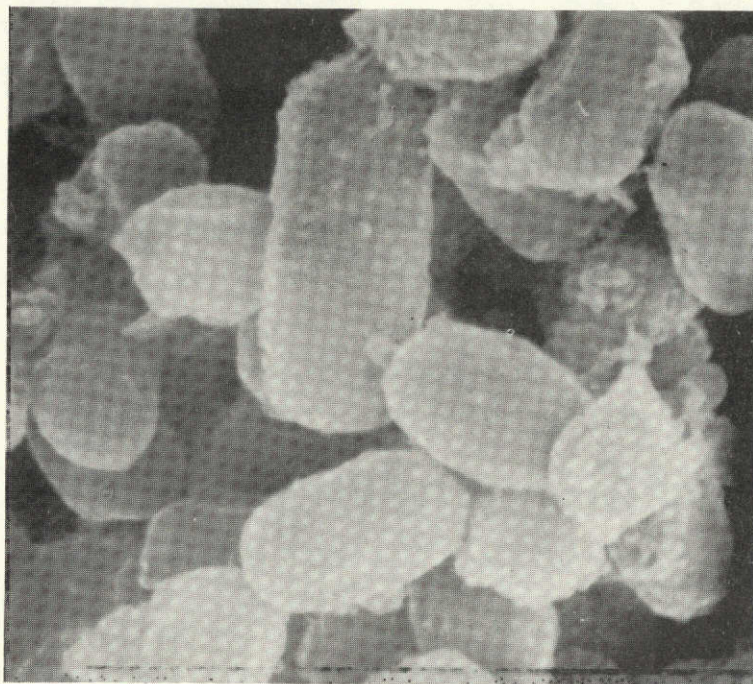


Figure 21b. Bacillus subtilis var. niger  
heated in buffer for 30 min.  
at 90° C.

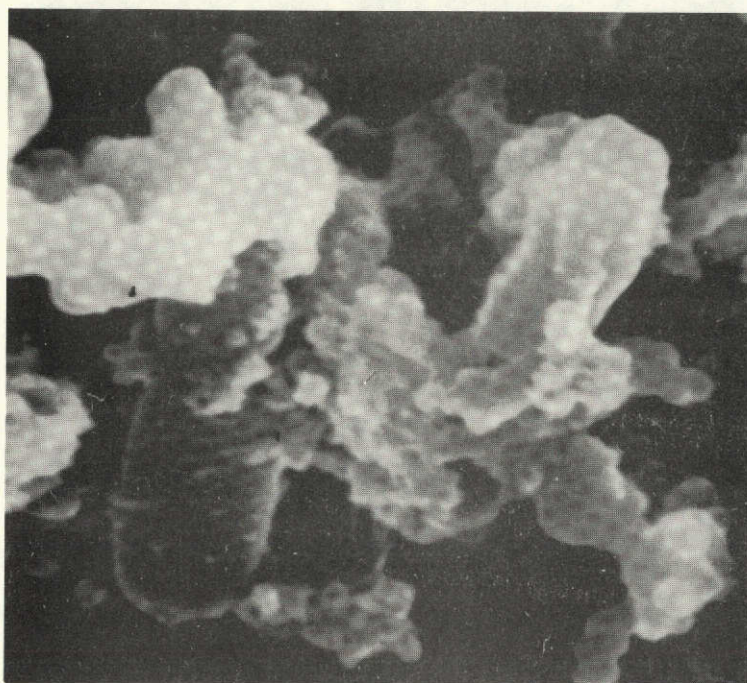


Figure 21c. Bacillus subtilis var. niger  
heated in buffer for 240 min.  
at 90° C.

## References

1. Angelotti, R., J. H. Maryanski, T. F. Butler, J. T. Peeler, and J. E. Campbell. 1968. Influence of spore moisture content on the dry-heat resistance of Bacillus subtilis var. niger. Appl. Microbiol. 16:735-745.
2. Black, S. H., and P. Gerhardt. 1962. Permeability of bacterial spores. III. Permeation relative to germination. J. Bacteriol. 83:301.
3. Campbell, L. L., and H. O. Halvorsen. 1965. Spores III. Amer. Soc. for Microbiol, 115 Huron View Blvd., Ann Arbor, Michigan.
4. Campbell, L. L. 1969. Spores IV. Amer. Soc. for Microbiol., 4715 Corbell Ave., Bethesda, Maryland 20014.
5. Curran, H. R., B. C. Brunstetter, and A. T. Myers. 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bacteriol. 45:485-494.
6. Curran, H. R., and F. R. Evans. 1945. Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol. 49:335-346.
7. Evans, F. R., and H. R. Curran. 1943. The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J. Bacteriol. 46:513-523.
8. Fox, K., and B. D. Eder. 1969. Comparison of survivor curves of Bacillus subtilis spores subjected to wet and dry heat. J. of Food Science. 34:518-521.
9. Gould, G. W., and A. Hurst. 1969. The bacterial spore. Academic Press, Inc., 111 Fifth Ave., New York, N.Y. 100013.
10. Halvorson, H. O., R. Hanson, and L. L. Campbell. 1972. Spores V. Amer. Soc. for Microbiol., 1913 First St., N.W., Washington, D.C. 20006.

11. Harnulv, B. G., and B. G. Snugg. 1972. Heat resistance of Bacillus subtilis spores at various water activities. J. Appl. Bacteriol. 35: 615-624.
12. Hitchins, A. D., G. W. Gould, and A. Hurst. 1963. The swelling of bacterial spores during germination and outgrowth. J. Gen. Microbiol. 30:445-458.
13. Hoffman, R. K., V. M. Gambill, and L. M. Buchanan. 1968. Effect of cell moisture on the thermal inactivation rate of bacterial spores. Appl. Microbiol. 16:1240-1244.
14. Knaysi, G. 1945. Investigation of the existence and nature of reserve material in the endospore of a strain of Bacillus mycoides by an indirect method. J. Bacteriol. 49:617-622.
15. Knaysi, G. 1957. Structure of the endospore and the cytological processes involved in its formation and germination, with remarks on criteria of germination. J. Appl. Bacteriol. 20(3):425-430.
16. Knaysi, G. 1965. Further observations on the spodogram of Bacillus cereus endospores. 90:453.
17. Lewis, J. C., H. S. Snell, and G. Alderton. 1965. Dormancy and activation of bacterial spores. In, Spores III, eds. L. L. Campbell and H. O. Halvorson, Minneapolis, Minn.: American Society for Microbiology.
18. Lechorvich, R. V. 1958. Thesis, University of Illinois.
19. Licciardello, J. J., and J. T. R. Nickerson. 1963. Some observations on bacterial thermal death time curves. J. Appl. Microbiol. 11:476-480.
20. Lin, P. S. 1973. Personal communication. Radiobiology Division, Tufts Medical Center, Boston, Mass.

21. Mayall, B. H., and C. Robinson. 1957. Observations with the electron microscope on the organization of the cortex of resting and germinating spores of B. megaterium. J. Appl. Bacteriol. 20(3):333-341.
22. Mikolajcik, E. M. 1971. Final report. Significance of spore-forming microorganisms in milk. Ohio State University Research Foundation #1040. FD-00072.
23. Moberly, B. J., F. Shafer, and P. Gerhardt. 1966. Structural details of anthrax spores during stages of transformation into vegetative cells. J. Bacteriol. 92:220.
24. Murrell, W. G., and A. D. Warth. 1965. In, Spores III, eds. L. L. Campbell and H. O. Halvorson. Amer. Soc. Microbiol., Ann Arbor, Michigan.
25. Murrell, W. G., and W. S. Scott. 1966. The heat resistance of bacterial spores at various water activities. J. Gen. Microbiol. 43: 411-425.
26. Powell, J. F. 1950. Factors affecting the germination of thick suspensions of Bacillus subtilis spores in L-alanine solution. J. Gen. Microbiol. 4:330.
27. Powell, J. F. 1953. Isolation of dypicolinic acid (pyridine-2:6-dicarboxylic acid) from spores of Bacillus megaterium. Biochem. J. 54:210.
28. Powell, J. F., and R. E. Strange. 1953. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. 54:205.
29. Powell, E. O. 1957. The appearance of bacterial spores under phase contrast illumination. J. Appl. Bacteriol. 20(3):342-348.
30. Powell, J. A. 1957. Biochemical changes occurring during spore germination in Bacillus species. J. Appl. Bacteriol. 20(3):349-358.



31. Pulvertaft, R. J. V., and J. A. Haynes. 1951. Adenosene and spore germination: Phase contrast studies. *J. Gen. Microbiol.* 5:657-663.
32. Rahn, O. 1945. Physical methods of sterilization of microorganisms. *Bacteriol. Rev.* 9:1.
33. Robinon, C. F. 1960. Morphology of bacterial spores, their development and germination. In, *The Bacteria I Structure*, eds. I. C. Gunsalus and R. Y. Stanier, Academic Press, New York, N.Y. and London, Chapt. 5, p. 207.
34. Spacecraft Sterilization Technology. 1966. N.A.S.A. Sp-108. U.S. Government Printing Office, Washington, D.C. 20402.
35. Strange, R. E., and F. A. Dark. 1957. A cell-wall lytic enzyme associated with spores of Bacillus species. *J. Gen. Microbiol.* 16:236-249.
36. Stumbo, C. R. 1965. *Thermobacteriology in food processing*. Academic Press, New York and London.
37. Sugiyama, H. 1951. Studies on factors affecting the heat resistance of spores of Clostridium botulinum. *J. Bacteriol.* 62:81-96.
38. Uehara, M., and H. A. Frank. 1967. Sequence of events during germination of putrefactive anaerobe 3679 spores. *J. Bacteriol.* 94:3, 506-511.
39. Vinter, V. 1962. In, *Spores II*. H. O. Halvorson, ed., p. 127, Burgess Publ. Co., Minneapolis, Minn.
40. Watkins, J. H., and C-E. A. Winslow. 1932. Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. *J. Bacteriol.* 24:243.
41. Woese, C. R., J. C. Vary,, and H. O. Halvorson. 1968. A kinetic model for bacterial spore germination. *Biochemistry.* 59:869-875.

42. Wolf, J., and C. M. Thorley. 1957. The effects of various germination agents on the spores of some strains of B. subtilis. J. Appl. Bacteriol. 20(3):384-389.
43. Manual of Methods for Pure Culture Study of Bacteria. 1946. Ed., The Committee on Bacteriological Technic of the Society of American Bacteriologists, Geneva, New York.